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Relative Efficacy of Two Probiotics in Controlling the Epizootic Ulcerative Syndrome Disease in Mrigal (*Cirrhinus mrigala* Ham.)

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ABSTRACT

The Epizootic Ulcerative Syndrome (EUS) is a dreaded disease of mrigal (*Cirrhinus mrigala* Ham.) inflicting heavy mortality in the latter fish at the farmers' ponds. To realize a good harvest, the control of this disease through the use of some probiotics is most important. The aim of study was to ascertain the effect of two probiotics on various blood and life parameters of mrigal (*C. mrigala*). Two commercially available probiotics with different compositions were tested for this purpose. *In vitro* trials were performed by the usual 'well poisoning method' whereas in *vivo* trials were performed in the fishes that were subjected to induced pathogenicity. In the treated fishes, periodic observations were recorded on their different hematological (viz., amount of hemoglobin, total erythrocyte count and total leukocyte count), survival and growth (length and weight) parameters. In the *in vitro* trials both probiotics developed clear zones of inhibition. However, the zone shown by probiotic-1 (having bacteria only) was smaller in size than that by probiotic-2 (in addition to bacteria also having vitamins, enzymes and salts), revealing the probable greater efficacy of the latter than the former. In the *in vivo* trials, the values of all the hematological parameters were found to decrease (leukocyte count increased) in the fish having induced pathogenicity and so were survival and growth parameters. But these values showed significant increase (leukocyte count decreased) in the probiotic treated fishes. This confirmed the useful role of both the probiotics in controlling the EUS disease in the mrigal. However, probiotic-2 was found to be more effective than probiotic-1 in increasing the hematological parameters and growth and survival in this fish.

Key words: Probiotic, epizootic ulcerative syndrome, disease, mrigal, *Cirrhinus mrigala*

INTRODUCTION

Probiotics have been characterized as new ecofriendly alternative measures of disease control in aquaculture (Irianto and Austin, 2002a, b; Dahiya *et al*., 2012a, b; Sihag and Sharma, 2012). Several microalgae, yeasts and gram-positive and gram-negative bacteria have been isolated from the aquatic medium (Van Damme and Vandepitte, 1980; Cahill, 1990; Liu *et al*., 2000; Alcaide, 2003; Austin, 2006; Dahiya and Sihag, 2009; Dahiya *et al*., 2009). Inhibition of pathogens in the digestive tract by the probiotic bacteria has been reported by several authors (Bogut *et al*., 1998; Yazid *et al*., 1999; Irianto and Austin, 2002a; Kabir *et al*., 2005; Trachoo and Boudreaux, 2006; Anukam, 2007; Nenci *et al*., 2007; Raj *et al*., 2008; Hung *et al*., 2008; Radfar and Farhoomand, 2008; Capcarova *et al*., 2008; Soundarapandian and Sankar, 2008; Vamanu *et al*.,

2008; Vijayabaskar and Somasundaram, 2008; Abdelhamid *et al*., 2009; Vali, 2009; Vamanu and Vamanu, 2010; Bansal *et al*., 2011; Dahiya *et al*., 2012a, b; Sihag and Sharma, 2012).

There are many examples of use of probiotics, directly or indirectly, preventing/controlling the diseases in aquatic animals (Stanier *et al*., 1993). For example, an isolate of *Micrococcus luteus* was found to combat *A. salmonicida* infections in rainbow trout (*Oncorhynchus mykiss*) (Irianto and Austin, 2002a); an enhanced fish growth was reported in the turbot (*Scophthalmus maximus*) when *Lactobacillus plantarum* was used as a probiotic (Gatesoupe, 1991); an administration of *Carnobacterium divergens* to the fry of Atlantic cod (*Gadus morhua* L.) was found to develop resistance in this fish against *Vibrio anguillarum* (Gildberg *et al*., 1997), *Bacillus toyoi* and *Enterococcus faecium* were found to be useful in reducing the edwardsiellosis in European eel (*Anguilla anguilla*) (Chang and Liu, 2002); *Pseudomonas fluorescens* was reported to inhibit *Sprolegnia,* and *A. salmonicida* in finfish culture (Smith and Davey, 1993; Bly *et al*., 1997). Even *A. hydrophila* and *V. fluvialis* were found to be effective in controlling the infections caused by *A. salmonicida* in rainbow trout (Irianto and Austin, 2002a). Bacteria and yeasts were used as probiotics in *Catla catla* to enhance its survival and body weight (Mohanty *et al*., 1996). *Bacillus subtilis* has been isolated from the rearing water of the common snook (*Centropomus undecimalis*) where it suppressed the growth of *Vibrio* spp. in the rearing water (Kennedy *et al*., 1998). This suggests that the bacteria may be suitable for biocontrol of the culture microflora of fish. The major taxonomic groups contributing to the healthy intestinal flora of fish species include *Vibrio, Lactobacillus, Acinetobacter* and *Achromobacter*, followed by *Micrococcus*, *Bacillus* and representatives from the family Enterobacteriaceae (Liston, 1957; Colwell, 1962; Ringo and Strom, 1994). In aquaculture, non-pathogenic strains of identified pathogenic bacteria have been successfully used as probiotics to control the diseases in fish (Austin *et al*., 1995; Gomez-Gil *et al*., 2002; Chythanya *et al*., 2002). Due to the presence of pathogenic and opportunistic bacteria in the environment, these organisms need to be suppressed to reduce their mucus proliferation and consequently the incidence of disease.

Fish under intensive culture conditions, are badly affected and are often infected by different microbial pathogens that have been treated with chemotherapeutic substances of which antibiotics are intensively used. These curative substances produce the problem against the action of bacterial drug 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 (Sihag and Sharma, 2012). Natural immunostimulants are biocompatible, biodegradable and safe for both the environment and human health. Moreover, they possess an added nutritional value. The use of biological products namely the probiotic is recently the goal of the disease biocontrol strategy in aquaculture as they improve the fish health and modify the fish associated microbial community.

The mrigal (*C. mrigala*) is one of the most important Indian major carps and is an integral part of the inland fisheries. In fact, this is an important component of sustainable food security in India. This fish was found to be infected with wide variety of diseases, including the infamous Epizootic Ulcerative Syndrome (EUS) which cause heavy mortality at fish-farmers' ponds (Sharma, 2009). Our earlier study revealed that six bacteria (viz. *Streptococcus faecalis, Aeromonas hydrophila, Streptococcus* grp Q1, *Cellobiosococcus sciuri, Shigella* sp. and *Micrococcus luteus*) and a fungus (*Aphanomyces invadans*) were responsible to cause EUS disease in the mrigal (Sharma *et al*., 2011). To alleviate the fish-farmers from the losses to be caused by this disease, its control measures

are essential. For this purpose and in the 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 two probiotics on various blood and life parameters of mrigal (*C. mrigala*).

MATERIALS AND METHODS

Two commercially available probiotics viz. probiotic-1 and probiotic-2 were tested for their role as disease controlling agents against the infections caused by pathogenic bacteria and fungi in mrigal (*C. mrigala*). These probiotics had the following organisms/food supplements:

Probiotic-1: This probiotic contained a complex of many bacteria viz. *Azospirillum, Bacillus licheniformis, Bacillus megaterium, Bacillus subtilis, Chlorobium, Disulpho vibrio sulphuriom, Nitrosomonas, Nitrobacter, Rhodobacter, Schizophyllum commune, Sclerotium glicanicum* and *Trichoderma.*

Probiotic-2: This probiotic had following bacteria and other food ingredients:

- **Bacteria:** *Bacillus subtilis, Bacillus licheniformis, Lactobacillus sporogenes, Lactobacillus acidophilus* and *Saccharomyces cerevisiae*
- **Sea weed extract:** Unspecified
- **Enzymes:** Amylase, beta-galactosidase, cellulase, lipase, phytase and protease
- **Vitamins:** Vitamin B6 = 1 g, Vitamin C = 20 g
- **Salts:** Sodium benzoate

***In vitro* tests on the role of probiotics inhibiting the growth of pathogenic bacteria:** *In vitro* tests of available probiotics for their antagonistic potential against bacteria and fungus were done by using poisoned food technique (Verma *et al.*, 2001; Jakhar *et al.*, 2010). For this purpose, separately fresh culture of each of the six bacteria (viz. *Streptococcus faecalis, Aeromonas hydrophila, Streptococcus* grp Q1, *Cellobiosococcus sciuri, Shigella* sp. and *Micrococcus luteus*) in nutrient broth was used. The causative nature of these organisms to induce Epizootic Ulcerative Syndrome (EUS) disease in the mrigal has already been tested by Sharma (2009). Cell free extract of each bacterium was prepared as described by Verma *et al.* (2001) and Jakhar *et al.* (2010). This cell free extract was tested for the antibacterial activity with the poisoned food technique. The experiment was replicated in four plates for each bacterium and the zones of inhibitions were measured. The results so recorded were compared statistically using paired t-test (Snedecor and Cochran, 1989).

***In vivo* tests on the role of probiotics inhibiting the growth of pathogenic bacteria:** The healthy individuals of mrigal fish weighing 20 g were brought from the fish farms of this study to the laboratory and were acclimated at 25°C for one week in a large porcelain tank of 30l capacity. The fish were fed a normal recommended commercial diet (Sobo fish feed, containing 35% protein, 4% fat, 3% fiber and 10% moisture, was given daily at the rate of 10% of the body weight of the total fishes in an aquarium measuring 24×12×12"). Only the healthy fishes showing normal activities were selected for further experimentation.

Table 1: Composition of different treatments administered to the experimental fishes

Treatment	Composition*
Control	Buffer saline alone
Bacterium alone	5×10^{11} CFU (colony forming units) mL^{-1} of bacterium
Fungus alone	100 spores of the fungus as determined by utilizing hemocytometer
Bacterium+fungus	5×10^{11} CFU mL^{-1} of bacterium+100 spores of the fungus
Bacterium+probiotic-1	5×10^{11} CFU mL^{-1} of bacterium+0.1 g of probiotic-1
Fungus+probiotic-1	100 spores of the fungus+0.1 g of probiotic-1
Bacterium+fungus+probiotic-1	5×10^{11} CFU mL^{-1} of bacterium+100 spores of the fungus+0.1 g of probiotic-1
Bacterium+probiotic-2	5×10^{11} CFU mL^{-1} of bacterium+0.1 g of probiotic-2
Fungus+probiotic-2	100 spores of the fungus+0.1 g of probiotic-2
Bacterium+fungus+probiotic-2	5×10^{11} CFU mL^{-1} of bacterium+100 spores of the fungus+0.1 g of probiotic-2
Probiotic-1 alone	0.1 g of probiotic-1
Probiotic-2 alone	0.1 g of probiotic-2

*A dose of 250 μL physiological saline buffer was inoculated into the intra-peritoneal cavity of the experimental fish which contained the respective ingredients

For a treatment, nine acclimated fish were taken randomly. *In vivo* pathogenicity test was carried out following the methods of Keskin *et al*. (2004). *Aeromonas hydrophila* (bacterium) and *Aphanomyces invadans* (fungus) were taken as pathogenic organisms for inoculation in the mrigal (*C. mrigala*). The causative nature of these organisms to induce EUS disease in the mrigal was earlier tested by Sharma (2009). The composition of each treatment is shown in Table 1.

Each dose dissolved in 250 μL of physiological buffer saline was inoculated into the intra-peritoneal cavity of the experimental fish. Three replicates of each treatment were used in the experiment. One fish from each replicate of ā treatment was sacrificed at weekly interval. The bacterial flora from each treatment and replicate was isolated and identified and the viable counts of the bacterial pathogens were worked out and recorded. The following parameters were recorded from the treated fish.

Levels of hematological parameters of the mrigal under different treatments: 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 (Dahiya *et al*., 2012a,b).

Under each treatment, the periodic levels of different blood parameters viz. total hemoglobin, total erythrocyte count and total leucocytes count were determined with the help of a haemocytometer and calculated from the equations given by Anderson and Klontz (1965).

Survival of the mrigal under different treatments: Survival of inoculated fish was determined with the help of total number of fish taken and number of fish that died using following formula:

$$S(\%)=\frac{N-M}{N}\times100$$

where, S is the survival, N is the total number of fish and M is the number of fish died.

Growth performance of the mrigal under different treatments

Gain in fish length/weight: Per cent gain in body length/weight was determined with the help of initial and final length/weight of the experimental fish using following formula:

$$G_g\% = \frac{G_2 - G_1}{G_1} \times 100$$

where, G is the fish length or weight as the case may be, 1 is the initial value, 2 is the final value and g is the gain in the respective attribute.

Statistical analysis: The results so recorded were analyzed statistically using completely randomized design (Snedecor and Cochran, 1989). F-values were tested at 5% level of significance following Analysis of Variance (using F-test) and critical differences (CD) among means were derived to evaluate differences among different treatment means.

RESULTS

***In vitro* tests on the role of probiotics inhibiting the growth of pathogenic bacteria:** Inhibition zones of the two probiotics against each bacterium were found to be different (Fig. 1a-f).

Probiotic-2 showed bigger inhibition zones as compared to probiotic-1 against each bacterium. From these results, it seemed that probiotic-2 was better than probiotic-1 in flushing out of pathogenic bacteria from the diseased fish. Probiotic-2 produced significantly bigger inhibition zones than those produced by probiotic-1 ($p \leq 0.05$, t-test).

***In vivo* tests on the role of probiotics inhibiting the growth of pathogenic bacteria**

Haemoglobin level in the blood of mrigal 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 2. The hemoglobin level of normal fish remained in the range of 6.27 to 6.55 g 100 mL^{-1}. However, in fishes inoculated with pathogenic bacterium and fungus alone, the level of hemoglobin fell drastically from 4.37 to 2.47 and 4.17 to 2.34 g 100 mL^{-1}, respectively. The hemoglobin level further declined from 4.06 to 2.13 g 100 mL^{-1} in fishes inoculated with bacterium along with fungus.

The hemoglobin level increased from 4.90 to 6.63 and 4.91 to 6.62 g 100 mL^{-1} in fish inoculated with bacterium+probiotic-1 and fungus+probiotic-1, respectively. The hemoglobin level increased from 4.41 to 6.60 g 100 mL^{-1} in fish inoculated with bacteria+fungus+probiotic-1 together. However, the hemoglobin level increased from 5.53 to 6.95 and 5.40 to 6.91 g 100 mL^{-1} in fish inoculated with bacteria+probiotic-2 and fungus+probiotic-2, respectively and from 4.91 to 6.61 g 100 mL^{-1} in fish inoculated with bacteria+fungus+probiotic-2. On the other hand, the fish given the treatment of probiotics (probiotic-1 and probiotic-2) showed maximal value of hemoglobin level as compared to all other treatments including control. The hemoglobin level increased from 5.77 to 7.10 g 100 mL^{-1} in fish administrated with probiotic-1 and from 6.67 to 7.35 g 100 mL^{-1} in fish administrated with probiotic-2.

The statistical analysis revealed that the effects of bacterium and fungus were similar on the level of hemoglobin in mrigal as the differences between the two means under these treatments (alone or in combination with the probiotics) over the study period were non-significant ($p \leq 0.05$; ANOVA, Table 2). However, both these pathogens together produced significantly more adverse

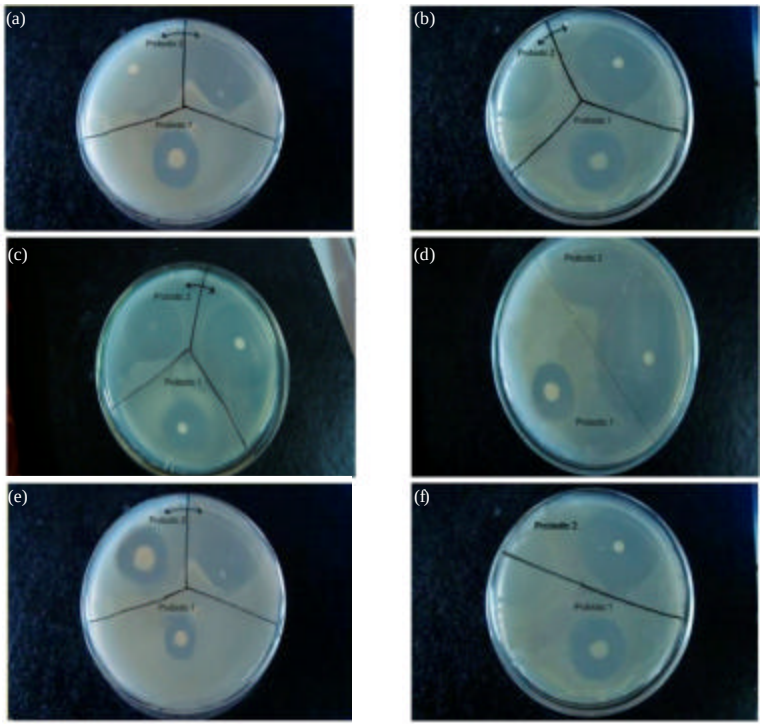

Fig. 1(a-f): Inhibition zones (diameter) of probiotic 1 and probiotic 2 against (a): *Streptococcus faecalis* (17±2.25 and 23±1.75 mm), (b): *Aeromonas hydrophila* (15±1.25 and 19±2.0 mm), (c): *Streptococcus* grp Q1 (18±2.0 and 23±1.75 mm), (d): *Cellobiosococcus sciuri* (16±2.0 and 22±1.5 mm), (e): *Shigella* sp. (16±2.0 and 21±1.0 mm) and (f): *Micrococcus luteus* (18±1.75 and 23±1.5 mm), the difference between the two inhibition zones (i.e. created by probiotic-1 and probiotic-2) was significant (based on paired t-test, $p<0.05$, 22 degrees of freedom)

Table 2: Effect of probiotics on the hemoglobin level of mrigal (*C. mrigala*) under *in vivo* induced pathogenicity over a period of eight weeks

	Hemoglobin (g 100 mL^{-1})							
	Weeks							
Treatment	1	2	3	4	5	6	7	8
Control	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
Bacterium alone	4.37±0.45	3.60±0.26	2.47±0.21	-	-	-	-	-
Fungus alone	4.17±0.26	3.54±0.22	2.34±0.12	-	-	-	-	-
Bacterium+fungus	4.06±0.08	3.48±0.17	2.13±0.29	-	-	-	-	-
Bacterium+probiotic-1	4.91±0.22	5.13±0.08	5.26±0.08	5.41±0.10	5.95±0.09	6.00±0.07	6.53±0.08	6.63±0.06
Fungus+probiotic-1	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
Bacterium+fungus+probiotic-1	4.41±0.05	4.55±0.05	5.00±0.02	5.15±0.03	5.41±0.05	6.13±0.06	6.31±0.07	6.60±0.04
Bacterium+probiotic-2	5.53±0.12	5.77±0.19	5.90±0.14	6.09±0.13	6.15±0.13	6.45±0.16	6.71±0.02	6.95±0.04
Fungus+probiotic-2	5.40±0.16	5.70±0.16	6.06±0.04	6.18±0.07	6.23±0.06	6.41±0.05	6.70±0.11	6.91±0.03
Bacterium+fungus+probiotic-2	4.91±0.22	5.16±0.06	5.31±0.10	5.46±0.09	6.00±0.05	6.15±0.03	6.45±0.05	6.61±0.06
Probiotic-1 alone	5.77±0.12	5.69±0.46	5.91±0.46	6.32±0.03	6.35±0.05	6.61±0.16	6.83±0.10	7.10±0.08
Probiotic-2 alone	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
CD value ($p \leq 0.01$)	0.417	0.403	0.405	0.916	0.175	0.133	0.168	0.079

Values are Mean±SD, N = 27 (9 fishes×3 replication), -: Fish died after three weeks, CD = Critical difference

Table 3: Effect of probiotics on the erythrocyte count of mrigal (*C. mrigala*) under *in vivo* induced pathogenicity over a period of eight weeks

	Erythrocyte count ($\times10^6$ cells mL^{-1})							
	Weeks							
Treatment	1	2	3	4	5	6	7	8
Control	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
Bacterium alone	1.25±0.06	1.18±0.01	1.16±0.01	-	-	-	-	-
Fungus alone	1.23±0.05	1.18±0.03	1.03±0.08	-	-	-	-	-
Bacterium+fungus	1.12±0.08	0.96±0.08	0.84±0.14	-	-	-	-	-
Bacterium+probiotic-1	1.41±0.03	1.49±0.02	1.54±0.02	1.58±0.02	1.66±0.04	1.87±0.05	2.21±0.09	2.51±0.07
Fungus+probiotic-1	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
Bacterium+fungus+probiotic-1	1.28±0.03	1.30±0.03	1.38±0.03	1.40±0.03	1.52±0.03	1.72±0.03	2.14±0.05	2.47±0.12
Bacterium+probiotic-2	1.58±0.04	1.64±0.04	1.68±0.03	1.75±0.04	2.15±0.02	2.31±0.03	2.65±0.07	2.67±0.05
Fungus+probiotic-2	1.49±0.07	1.54±0.08	1.59±0.10	1.66±0.08	1.72±0.05	1.90±0.11	2.30±0.11	2.58±0.04
Bacterium+fungus+probiotic-2	1.37±0.12	1.41±0.11	1.48±0.09	1.58±0.02	1.80±0.02	2.14±0.05	2.31±0.10	2.53±0.11
Probiotic-1 alone	2.28±0.11	2.33±0.11	2.36±0.10	2.42±0.05	2.59±0.04	2.64±0.02	2.64±0.01	2.74±0.02
Probiotic-2 alone	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
CD value ($p \leq 0.01$)	0.11	0.10	0.12	0.19	0.06	0.08	0.11	0.10

Values are Mean±SD, N = 27 (9 fishes×3 replication), -: Fish died after three weeks, CD: Critical difference

effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 2). Addition of probiotics could significantly increase the level of hemoglobin in the respective treatment versus time combination. However, increase due to probiotic-2 was significantly more than probiotic-1 ($p \leq 0.05$, ANOVA, Table 2). It was, therefore, evident that probiotic-2 gave better results in increasing the hemoglobin level of fish as compared to probiotic-1.

Total erythrocyte count in the blood of mrigal 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 3. The erythrocyte count level of normal fish remained in the range of 2.21 to 2.25×10^6 cells mL^{-1}. However, in fishes inoculated with pathogenic bacterium and fungus alone, the level of erythrocyte count fell drastically and decreased from 1.25 to 1.16×10^6 cells mL^{-1} and 1.23 to 1.03×10^6 cells mL^{-1}, respectively. The level of erythrocyte count further declined from 1.12 to 0.84×10^6 cells mL^{-1} in fishes treated with bacterium along with fungus.

The erythrocyte count level increased from 1.41 to 2.51×10^6 cells mL^{-1} and 1.32 to 2.50×10^6 cells mL^{-1} in fish inoculated with bacterium+probiotic-1 and fungus+probiotic-1, respectively. The erythrocyte count level increased from 1.28 to 2.47×10^6 cells mL^{-1} in fish inoculated with bacteria+fungus+probiotic-1 together. However, the erythrocyte count level increased from 1.58 to 2.67×10^6 and 1.49 to 2.58×10^6 cells mL^{-1} in fish inoculated with bacteria+probiotic-2 and fungus+probiotic-2, respectively and from 1.37 to 2.53×10^6 cells mL^{-1} in fish inoculated with bacteria+fungus+probiotic-2.

On the other hand, the fish given the treatment of probiotics (probiotic-1 and probiotic-2) showed maximal value of erythrocyte count level as compared to all other treatments including control. The erythrocyte count level increased from 2.28 to 2.74×10^6 cells mL^{-1} in fish administrated with probiotic-1 and from 2.49 to 3.10×10^6 cells mL^{-1} in fish administrated with probiotic-2.

The statistical analysis revealed that the effects of bacterium and fungus on erythrocyte count of mrigal were similar as the differences between the two means under these treatments (alone or

Table 4: Effect of probiotics on the total leukocyte count of mrigal (*C. mrigala*) under *in vivo* induced pathogenicity over a period of eight weeks

	Leukocyte count ($\times 10^3$ mL^{-1})							
	Weeks							
Treatment	1	2	3	4	5	6	7	8
Control	2.29±0.15	2.34±0.13	2.35±0.24	2.33±0.12	2.36±0.07	2.39±0.09	2.34±0.04	2.35±0.03
Bacterium alone	3.20±0.08	3.80±0.16	4.00±0.40	-	-	-	-	-
Fungus alone	3.1±0.02	3.31±0.09	3.91±0.03	-	-	-	-	-
Bacterium+fungus	3.03±0.16	3.15±0.04	3.80±0.40	-	-	-	-	-
Bacterium+probiotic-1	3.10±0.08	3.05±0.06	2.91±0.06	2.85±0.05	2.76±0.05	2.68±0.04	2.52±0.02	2.48±0.03
Fungus+probiotic-1	2.94±0.12	2.83±0.07	2.79±0.06	2.73±0.06	2.67±0.08	2.62±0.07	2.52±0.06	2.46±0.04
Bacterium+fungus+probiotic-1	2.65±0.07	2.62±0.04	2.60±0.10	2.56±0.08	2.53±0.07	2.44±0.02	2.44±0.02	2.40±0.01
Bacterium+probiotic-2	3.19±0.01	3.09±0.02	3.06±0.03	3.09±0.06	3.17±0.03	3.23±0.05	3.01±0.04	2.92±0.08
Fungus+probiotic-2	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
Bacterium+fungus+probiotic-2	2.92±0.16	2.72±0.12	2.87±0.05	2.82±0.03	2.77±0.04	2.68±0.04	2.62±0.03	2.48±0.04
Probiotic-1 alone	2.39±0.36	2.28±0.19	2.22±0.08	2.14±0.08	2.14±0.19	2.10±0.18	2.02±0.16	1.85±0.10
Probiotic-2 alone	2.40±0.42	2.27±0.23	2.18±0.28	2.09±0.23	2.06±0.07	2.02±0.04	1.97±0.03	1.70±0.02
CD value ($p \leq 0.01$)	0.314	0.198	0.339	0.55	0.187	0.117	0.092	0.074

Values are Mean±SD, N = 27 (9 fishes×3 replication), -: Fish died after three weeks, CD: Critical difference

in combination with the probiotics) over the study period were non-significant ($p \geq 0.05$; ANOVA, Table 3). However, both these pathogens together produced significantly more adverse effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 3). Addition of probiotics could significantly increase the level of erythrocyte count in the respective treatment versus time combination. However, increase due to probiotic-2 was significantly more than probiotic-1 ($p \leq 0.05$, ANOVA, Table 3). It was, therefore, evident that probiotic-2 gave better results in increasing the erythrocyte count level of fish as compared to probiotic-1.

Total leukocyte count in the blood of mrigal 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 4. The leukocyte count exhibited an increase due to pathogenicity and its decrease after the administration of probiotics. The leukocyte count level of normal fish remained in the range of 2.29 to 2.39×10^3 cells mL^{-1}. However, in fishes inoculated with pathogenic bacterium and fungus alone, the level of leukocyte increased from 3.20 to 4.00×10^3 and 3.16 to 3.91×10^3 cells mL^{-1}, respectively. The level increased from 3.03 to 3.80×10^3 cells mL^{-1} in fishes treated with bacterium along with fungus.

The leukocyte count decreased from 3.10 to 2.48×10^3 and 2.94 to 2.46×10^3 cells mL^{-1} in fish inoculated with bacterium+probiotic-1 and fungus+probiotic-1, respectively. The level decreased from 2.65 to 2.40×10^3 cells mL^{-1} in fish inoculated with bacteria+fungus+probiotic-1 together. However, the leukocyte count level decreased from 3.19 to 2.92×10^3 and 3.18 to 2.53×10^3 cells mL^{-1} in fish inoculated with bacteria+probiotic-2 and fungus+probiotic-2, respectively and from 2.92 to 2.48×10^3 cells mL^{-1} in fish inoculated with bacteria+fungus+probiotic-2.

On the other hand, the fish given the treatment of probiotics (probiotic-1 and probiotic-2) showed minimal value of leukocyte count as compared to all other treatments including control. The level of leukocyte count decreased from 2.39 to 1.85×10^3 cells mL^{-1} in fish administrated with probiotic-1 and from 2.40 to 1.70×10^3 cells mL^{-1} in fish administrated with probiotic-2.

The statistical analysis revealed that the effects of bacterium and fungus on the leukocyte count of mrigal were similar as the differences between the two means under these treatments (alone or in combination with the probiotics) over the study period were non-significant ($p \leq 0.05$; ANOVA, Table 4). However, both these pathogens together produced significantly more adverse effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 4). Addition of probiotics could significantly decrease the level of leukocyte count of mrigal in the respective treatment versus time combination. However, decrease due to probiotic-2 was significantly more than probiotic-1 ($p \leq 0.05$, ANOVA, Table 4). It was, therefore, evident that probiotic-2 gave better results in increasing the level of leukocyte count of mrigal fish as compared to probiotic-1.

Survival of the mrigal under different treatments: The results on survival of mrigal under different treatments over a period of eight weeks are presented in Table 5. The survival of normal fish during this period was 100%. But in the fish inoculated with bacterium alone, fungus alone and bacterium+fungus together, the survival was only 29.6, 27.2 and 11.1%, respectively. Under these treatments, at the end of third week, all the fish died. The fish inoculated with bacteria along with fungus showed less survival as compared to other treatments as well as control. Other groups of fish which were given the treatments of probiotics (probiotic-1 and probiotic-2) showed 100% survival.

The statistical analysis revealed that the effects of bacterium and fungus on survival of mrigal were similar as the differences between the two means under these treatments (alone or in combination with the probiotics) over the study period were non-significant ($p \geq 0.05$; ANOVA, Table 5). However, both these pathogens together produced significantly more adverse effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 5). Addition of probiotics could significantly increase the survival of fish in the respective treatment versus time combination. When used, both the probiotics resulted in to cent per cent survival of the fish. However, on the basis of overall results, probiotic-2 gave better results in increasing the survival of fish as compared to probiotic-1.

Table 5: Effect of probiotics on the survival of mrigal (*C. mrigala*) under *in vivo* induced pathogenicity over a period of eight weeks

	Percent survival in different weeks		
Treatment	1	2	3
Control	100.0±0.00	100.0±0.00	100.0±0.00
Bacterium alone	85.0±3.73	40.7±3.70	29.6±0.00*
Fungus alone	82.8±3.66	37.0±3.70	27.2±3.70*
Bacterium+fungus	74.8±0.00	22.2±0.00	11.1±0.00*
Bacterium+probiotic-1	100.0±0.00	100.0±0.00	100.0±0.00
Fungus+probiotic-1	98.0±0.00	100.0±0.00	100.0±0.00
Bacterium+fungus+probiotic-1	89.0±0.00	100.0±0.00	100.0±0.00
Bacterium+probiotic-2	100.0±0.00	100.0±0.00	100.0±0.00
Fungus+probiotic-2	100.0±0.00	100.0±0.00	100.0±0.00
Bacterium+fungus+probiotic-2	100.0±0.00	100.0±0.00	100.0±0.00
Probiotic-1 alone	100.0±0.00	100.0±0.00	100.0±0.00
Probiotic-2 alone	100.0±0.00	100.0±0.00	100.0±0.00
CD value ($p \leq 0.01$)	4.9	4.4	3.1

Values are Mean±SD, N = 27 (9 fishes×3 replication), *Fish died after three weeks, CD: Critical difference

Table 6: Length gain in mrigal (*C. mrigala*) under different treatments over a period of eight weeks

	Length gain (cm)		
Treatment	Initial length	Final length	Increase (%)
Control	8.0±0.05	12.2±0.10	52.5
Bacterium alone	7.8±0.11	9.5±0.10	21.8*
Fungus alone	7.8±0.05	9.3±0.11	19.2*
Bacterium+fungus	8.0±0.07	8.6±0.08	7.5*
Bacterium+probiotic-1	8.2±0.05	13.3±0.12	62.2
Fungus+probiotic-1	8.0±0.02	12.6±0.19	57.5
Bacterium+fungus+probiotic-1	8.3±0.05	12.4±0.21	49.4
Bacterium+probiotic-2	8.3±0.06	14.9±0.08	79.5
Fungus+probiotic-2	7.9±0.12	14.0±0.05	77.2
Bacterium+fungus+probiotic-2	8.3±0.12	14.0±0.09	68.7
Probiotic-1 alone	7.5±0.06	13.0±0.08	73.3
Probiotic-2 alone	8.2±0.17	15.9±0.12	93.9

Values are Mean±SD, N = 27 (9 fishes×3 replication), *Fish died after three weeks, CD (initial length), $p>0.05 = 0.9$, CD (final length), $p<0.05 = 0.6$

Growth performance of the mrigal under different treatments

Gain in fish length: The results of length of mrigal (*C. mrigala*) under different treatments over a period of eight weeks are presented in Table 6. The fish under normal condition showed 52.5% increase in length; the increase in length of the fish inoculated with bacterium and fungus was only 21.8 and 19.2%, respectively. When the fish was inoculated with bacteria along with fungus, these showed still less increase in length (7.5%) indicating that fish growth is severely affected by the diseases and multiple infections put more severe effect as compared to single species infection. Use of probiotics seemed to suppress the fish disease which was indicated by the relative more increase in fish length in single species and mixed inoculation of pathogens along with probiotics. The increase in fish length was 62.2, 57.5 and 49.4% when probiotic-1 was administrated with bacterium alone, fungus alone and bacterium+fungus together respectively. The corresponding figures were 79.5 77.2 and 68.7, respectively when probiotic-2 was administrated. However, when probiotic-1 and probiotic-2 were administrated in healthy fish, the increase in length of fish was 73.3 and 93.9%.

The statistical analysis revealed that the effects of bacterium and fungus on gain in length of mrigal were similar as the differences between the two means under these treatments (alone or in combination with the probiotics) over the study period were non-significant ($p \leq 0.05$; ANOVA, Table 6). However, both these pathogens together produced significantly more adverse effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 6). Addition of probiotics could significantly increase the fish length in the respective treatment. However, increase in fish length due to probiotic-2 was significantly more than probiotic-1 ($p \leq 0.05$, ANOVA, Table 6). It was, therefore, evident that probiotic-2 gave better results in increasing the length of fish as compared to probiotic-1.

Gain in fish weight: The results of weight of mrigal (*C. mrigala*) under different treatments over a period of eight weeks are presented in Table 7. The fish under normal condition showed 326.3% increase in weight; the increase in weight of the fish inoculated with bacterium and fungus

Table 7: Weight gain in mrigal (*C. mrigala*) under different treatments over a period of eight weeks

	Weight gain (g)		
Treatment	Initial weight	Final weight	Increase (%)
Control	11.8±0.02	50.3±0.11	326.3
Bacterium alone	11.6±0.04	20.1±0.02	73.3*
Fungus alone	11.9±0.03	17.1±0.01	43.7*
Bacterium+fungus	12.2±0.03	13.9±0.05	13.9*
Bacterium+probiotic-1	11.9±0.05	62.0±0.08	421.0
Fungus+probiotic-1	11.9±0.04	59.1±0.17	405.0
Bacterium+fungus+probiotic-1	11.8±0.03	53.0±0.05	349.2
Bacterium+probiotic-2	12.2±0.01	65 .3±0.06	435.2
Fungus+probiotic-2	11.5±0.01	60.2±0.06	423.5
Bacterium+fungus+probiotic-2	11.5±0.03	57.1±0.03	396.5
Probiotic-1 alone	11.5±0.01	75.0±0.08	552.2
Probiotic-2 alone	12.1±0.02	89.0±0.12	635.5

Values are Mean±SD, N = 27 (9 fishes×3 replication), *Fish died after three weeks, CD (initial length), $p>0.05 = 0.9$, CD (final length), $p<0.05 = 3.1$

was only 73.3 and 43.7%, respectively. When the fish was inoculated with bacteria along with fungus, these showed still less increase in weight (13.9%) indicating that fish growth is severely affected by the diseases and multiple infections put more severe effect as compared to single species infection. Use of probiotics seemed to suppress the fish disease which was indicated by the relative more increase in fish weight in single species and mixed inoculation of pathogens along with probiotics. The increase in fish weight was 421, 405 and 349.2% when probiotic-1 was administrated with bacterium alone, fungus alone and bacterium+fungus together respectively. The corresponding figures were 435.2, 423.5 and 396.5, respectively when probiotic-2 was administrated. However, when probiotic-1 and probiotic-2 were administrated in healthy fish, the increase in weight of fish was 552.5 and 635.5%.

The statistical analysis revealed that the effects of bacterium and fungus on weight gain of mrigal were similar as the differences between the two means under these treatments (alone or in combination with the probiotics) over the study period were non-significant ($p \geq 0.05$; ANOVA, Table 7). However, both these pathogens together produced significantly more adverse effect as compared to any one of these acting alone ($p \leq 0.05$, ANOVA, Table 7). Addition of probiotics could significantly increase the weight in the respective treatment. However, increase due to probiotic-2 was significantly more than probiotic-1 ($p \leq 0.05$, ANOVA, Table 7). It was, therefore, evident that probiotic-2 gave better results in increasing the weight of fish as compared to probiotic-1.

DISCUSSION

Effect of pathogenic bacteria on hematological parameters of fish: Hematological parameters reflect the poor condition of fish more quickly than other commonly measured parameters. A number of hematological indices such as hemoglobin, red blood cells and white blood cells, packed cell volume and so on have been used to assess the functional status of oxygen carrying capacity and defense system of the blood stream which enhances the immune system (Chinabut *et al*., 1995). However, very scanty work has been done on these parameters in the fishes. The level of blood in the common carp decreased after exposure to the cyanobacterial extract

(Palikova *et al*., 2004), Erythrocytes count and haematocrit/packed cell volume of Nile tilapia decreased when inoculated with *Mycobacterium marinum* which may lead to a tendency to anemia (Ranzani-Paiva *et al*., 2004). Results of these studies resemble those of the present investigation which indicated that the mrigal fish inoculated with pathogenic bacteria and fungus showed a decrease in its blood parameters. Hemoglobin level reduced approximately to 55% (Table 2); erythrocytes count reduced approximately to 75-90% (Table 3) and leukocyte count increased to approximately 125% (Table 4) in three weeks. This clearly indicated a marked decline in the hemoglobin and erythrocyte counts and increase in the leukocyte counts of diseased fishes.

Effect of probiotic on hematological parameter: Probiotics have been reported to generate beneficial effects on health of the host. These beneficial effects include disease treatment and prevention as well as improvement of digestion and absorption in the host (Havenaar and Huis in't Veld, 1992). Fish fed on probiotic bacteria showed an increase in erythrocyte count than the control group (Irianto and Austin, 2002a). The probiotics used in carps increased the level of blood parameters as a result of hemopiotic stimulation when fed on probiotic bacteria (Lalloo *et al*., 2007). Irianto and Austin (2002a) used dead probiotic cells to control disease and observed higher number of leukocytes, erythrocytes and macrophages in rainbow trout, (*Oncorhynchus mykiss*). When *Bacillus* sp. was used as probiotics in the tiger shrimp (*Penaeus monodon*), the treated individuals were found to develop disease resistance and also increase in the level of their selected hematological parameters in the blood e.g. red blood cell count, hematocrit, hemoglobin and various leukocyte counts (Rengpipat *et al*., 2000; Siwicki *et al*., 1994). When the fish *Cyprinus carpio* was fed with fungus (*Saccharomyces cerevisiae*), an increase in its total leukocyte counts and in proportion of neutrophils and monocytes was observed (Selvaraj *et al*., 2005). The results of present study revealed that probiotic had a positive effect on hemoglobin level which increased approximately to 50% in its value (Table 2), erythrocytes count increased approximately to 40% in its value (Table 3) and leukocyte count decreased approximately to 30% in its value (Table 4). This clearly indicated that use of probiotics had positive effect on the hematological parameters of the mrigal fish.

Effect of probiotic on survival and growth of treated fishes: Survival of larvae of sea bass was significantly higher than the control when fed 1.1% live yeast as a probiotic (Tovar-Ramirez *et al*., 2004). Kennedy *et al*. (1998) also showed that the addition of a gram-positive probiotic bacterium increased the survival, size uniformity and growth rate of marine fish larvae (snook, red drum, spotted sea trout and stripped mullet). In the present study, the survival of normal fish during the experimental period was 100%. But in the fish inoculated with bacterium alone, fungus alone and bacterium+fungus together, the survival was only 29.6, 27.2 and 11.1%, respectively; at the end of third week, all these fish died. The fish inoculated with bacteria along with fungus showed least survival among all the treatments. The fish given the treatments of probiotics (probiotic-1 and probiotic-2) showed 100% survival (Table 5).

Probiotic incorporated feed had a definite role in enhancing the growth of channel catfish and turbot larvae (Gatesoupe, 1991). A significant increase in the growth of *Penaeus monodon* and *Penaeus vannamei* was reported when fed probiotic incorporated feeds (Maeda and Liao, 1992, 1994; Garriques and Arevalo, 1995). The probiotics were found to act as growth promoters in carps and other fishes (Noh *et al*.,1994; Gildberg *et al*., 1995, 1997; Rengpipat *et al*., 1998; Prabhu *et al*., 1999).

Selvaraj *et al.* (2005) also reported the enhanced immune response of β-glucan (extracted and purified from *Saccharomyces cerevisiae*) administered in *C. carpio* on day 1, 3 and 5 through different route (intraperitoneally, bathing and orally). They also reported enhanced percent survival significantly when 500 μg of β-glucan was injected intra-peritoneally, but bathing and oral administration did not show any influence. Likewise, *Streptococcus faecium* was found to improve the growth and feeding efficiency of carp (Noh *et al.*, 1994; Bogut *et al.*, 2000; Irianto and Austin, 2002a) by stimulating appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet and by the breakdown of indigestible components. Several probiotic bacterial species including *Lactobacillus* sp. (Jonsoon, 1986) and mixed cultures of different bacteria (Lessard and Brisson, 1987) were used to improve the nutrition level and immunity of aquacultural animals against pathogenic microorganisms. In addition, the use of antibiotics can be reduced and frequent outbreaks of diseases can be prevented. Lactic acid bacteria had an effect as growth promoter on the growth rate in carps (Noh *et al.*, 1994). Also, *Enterococcus faecium* has been used as probiotic to improve growth when fed to sheatfish, *Silurus glanis* L. (Bogut *et al.*, 2000). Some naturally occurring bacteria were found to be able to promote the growth and survival of oyster (*Argopecten purpuratus)* larvae by inhibiting the activity of other bacteria that flourish in hatchery cultures (Riquelme *et al.*, 1997).

In the present study, the length gain on the 60th day of the experiment was found to be maximal in the mrigal fishes fed probiotic-2 which was 93.9% increase over the initial length. On the other hand the fishes fed probiotic-1 showed 79.3% increase in length compared to 51.9 percent of the control (Table 6). Likewise, the weight gain on the 60th day of the experiment was found to be maximal in fishes fed probiotic-2 which was 635.5% compared to 552.2% in the fishes fed with probiotic-1 and 365.7% in the control fishes (Table 7). A significant difference in growth was observed between the probiotic treated fishes and the control ($p<0.05$, ANOVA, Table 6, 7). The survival rate of fish increased and reached up to 100% in the probiotic treated fishes. Over all, length and weight gain and survival were found to increase in mrigal (*C. mrigala*) fed a diet containing probiotic.

Role of other components present in probiotic-2 along with bacteria

Pigments and vitamins: The production of inhibitory metabolites by bacteria appears to correlate with the expression of pigments (Holmstrom *et al.*, 2002; Egan *et al.*, 2002) and additionally, pigments in the form of carotenoids are important in the production of various vitamins (Ronnestad and Lie, 1998). For example, the vitamin A in the eyes of halibut (*Hippoglossus hippoglossus*) is mainly derived from dietary carotenoids (Ronnestad *et al.*, 1998) compared to larvae fed *Artemia* which have lower levels of vitamin A (Ronnestad and Lie, 1998). Fish are unable to synthesize vitamin C and are dependent on a constant supply through their food (Chatterjee, 1973). Increasing the levels of vitamin C available to the larvae can be achieved through enrichment of the live food (Olsen *et al.*, 2000). The higher localized concentration of the vitamin in the *Penaeus monodon* helps increase the growth rate of prawn (Hancock and Viola, 2001).

Enzymes: Enzyme complex contain amylase, phytase, protease and lipase which are predominant and considered to be important during the early stages of fish development (Ribeiro *et al.*, 1999; Gawlicka *et al.*, 2000). Lipase, considered to be important in enhancement of growth of fish, is not commonly found in developing fish (Baglole *et al.*, 1998; Martinez *et al.*, 1999). This suggests that the enzymes may be suitable for the enhancement of growth of fish.

The purpose of parallel use of two probiotics was to check the relative effectiveness of these probiotics to control the disease and to ascertain the role of other components in the probiotics responsible for that. In the present study, the effect of probiotic-2 was better than probiotic-1. The former contained vitamins and enzymes along with bacteria. That may be the reason of better efficacy of probiotic-2 than probiotic-1 in controlling the EUS disease in the mrigal.

CONCLUSION

This study reveals that fishes treated with probiotics showed significant increase in the levels of different hematological parameters (viz. hemoglobin, erythrocyte count and leukocyte count) over the control fishes. The fishes having induced pathogenicity and subsequently treated with probiotics also showed increase in the levels of hematological parameters. There was significant increase in the growth rate (in the form of length and weight gain) and survival of the fishes given probiotics. The fishes having induced pathogenicity could not survive beyond three week. This study therefore, clearly reveals that probiotics are very effective in controlling the EUS diseases in the mrigal and in improving its health status. Probiotic-2, having many other essential ingredients, was more effective than the probiotic-1 in controlling the EUS disease in this fish.

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