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Research Article

Effect of Probiotics and Prebiotics Supplemented Diets on Immune Resistance against *Aeromonas hydrophila* in Mrigal Carp

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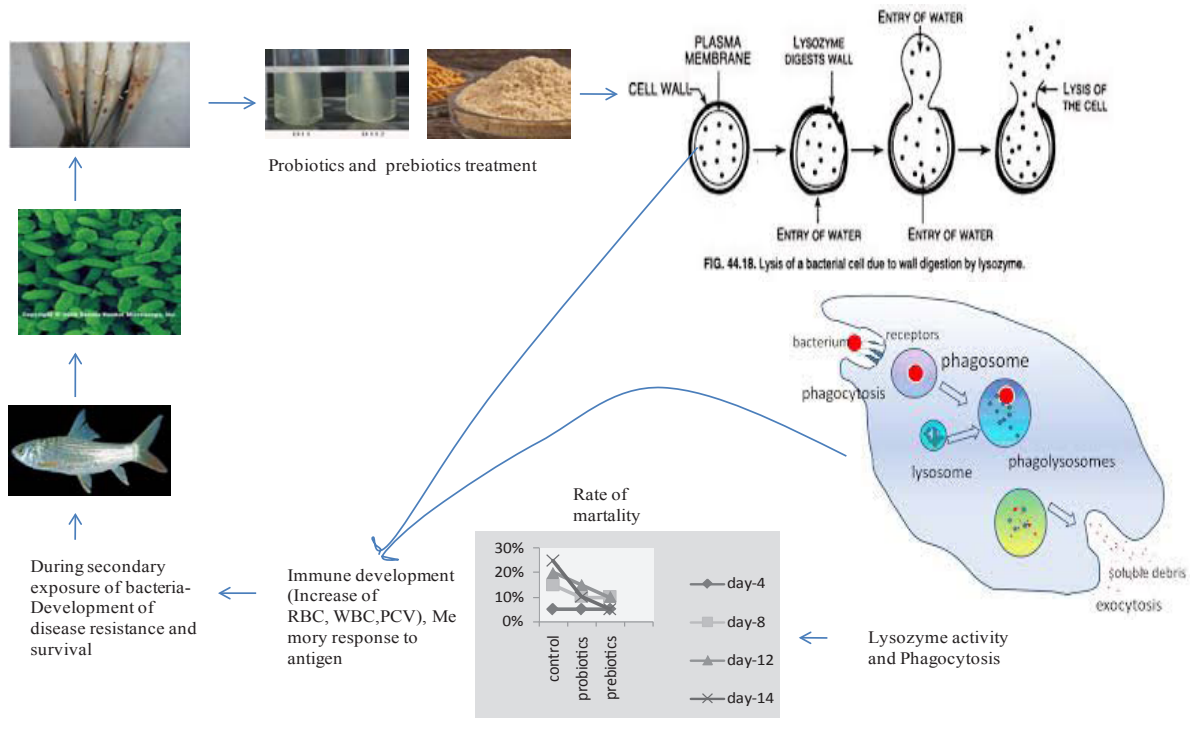
Keywords

- *Enterococcus faecalis*; *Aeromonas hydrophila*; Rice bran; Disease resistance; *Cirrhinus mrigala*

Abstract

The present study was made to test the effect of probiotics and prebiotics on disease resistance. Three treatments were designed including a control, probiotics (*Enterococcus faecalis*) and prebiotics (Rice bran) incorporated in the fish feed which was administrated for a period of 45 Days. Results showed that probiotics and prebiotics on oral administration resulted in improved Serum lysozyme count, Serum bactericidal activity, Serum protein level, Serum globulin level, Hematological parameters such as, Leukocyte count, Red blood cell count, and Packed cell volume. The mortality rate after challenge with *Aeromonas hydrophila* was significantly low in fish fed control-65%, probiotics-40%, prebiotics-30%. The present study suggests that probiotics and prebiotics supplemented diet enhances the disease resistance to *Aeromonas hydrophila* in *Cirrhinus mrigala*.

GRAPHICAL ABSTRACT



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INTRODUCTION

All over the world aquaculture has grown extremely during the last few years becoming an commercially important sector [1]. All over the world, aqua culture field is one of the fastest growing food-producing sector. The increased inflation of aquaculture has led to a high level of disease outbreaks with an increasing range of micro organisms causing them [2]. Currently, the function of the aquaculture industry is to boosting the growth, survival performance, feed efficiency, and resistance of aquatic pathogenic organisms, while reducing production amounts [3].

The word "pro" and "bios" comes from the Greek words. It means prebiotics ("before life'). A prebiotic was usually defined as "A non-digestible foods ingredient (s) that beneficially affects the host animal by selectively stimulating the action of single or a limited number of bacteria in the colon, and growth and they promote host animal health". According to [4-6], various food substances such as certain lipids, some proteins and peptides, non-digestible carbohydrates, act as prebiotic ingredient. Prebiotics are non-digestible food ingredient that stimulate the activity or growth of beneficial commensal bacteria in the gut of host organism thus improves host health level [5-7], reported that a food ingredient which acts as prebiotics must possess the following principle such as showing hydrolysis by digestive enzyme, fermentation by gastrointestinal micro flora resistance to gastric acidity, and increase the abundance of intestinal bacteria or micro organisms related to health development.

Some gram positive bacteria like Enterococcus, Bacillus, Streptococcus act as general probiotic strains which are the main gastrointestinal microbial organisms [8]. Probiotics are used in fish culture to improve nutrition [9], growth performance [10], decrease diseases [11] and develop immune system [12].

In recent years there has been high interest in the benefit of prebiotics in aquaculture [13]. Besides therapeutics and vaccines, an alternate approach to enhance disease resistance, immune responses and other health benefits is the application of probiotics, prebiotics and other feed additives which have different health promoting properties for carp species are encouraged [14-16]. The immunity of fish is physiologically similar to that of higher vertebrates, despite certain differences. In contrast to higher vertebrates, fish are free-living aquatic organisms from early embryonic stages of life and depend on their innate immunity for survival [17].

A. hydrophila and other *Aeromonas* species are among the most probable bacteria in fresh water culture systems, and these bacteria usually cause disease among feral and cultured fishes throughout the world [18-22].

A. hydrophila is one of the essential opportunistic bacterias of freshwater fish has been generally combined with the epizootic ulcerative syndrome which caused group of mortality to cultured and wild fish in various parts of South East Asia [23,24]. Species of *Aeromonas* are rod-shaped, non-spore-forming, Gram-negative, facultative, anaerobic bacteria that occur omnipresently and autochthonous in natural habitats such as soils and aquatic habitats [25]. *A. hydrophila* is a omnipresent rod-shaped, gram negative bacterium, has broad level of host susceptibility in common carp, koi carp, cat fish, and gold fish. *A. hydrophila* produces a wide variety of extra cellular products (ECP) including aerolysin, haemolysins, enterotoxin and cytotoxin [26,27]. *A. hydrophila* is the main causative organism of the ulcerative disease known as "haemorrhagic septicemia" that manifested as red skin sores disease [28]. Outbreaks of haemorrhagic ulceration disease are mainly noticed to show specially when temperature levels between 10°**C** and 20°C, causing mortality up to 100% with the involvement of pathogenic bacteria *A. Hydrophila* [29]. *Aeromonas hydrophila* along with *A. sobria* was regularly reported as a formative agent of motile *Aeromonas* septicemia (MAS) in fish as well as other aquatic animals [30-33]. This bacterium is a heterogeneous and ubiquitous organism that causes infection under stress conditions or in concert with infection by other pathogenic organisms. *A. hydrophila* is frequently associated with disease in eels, carps, channel catfish, milkfish, tilapia, ayu and trout [34]. The aim of this study to evaluate the effect of probiotics and prebiotics supplemented diets on disease resistance against *A. hydrophila* in *C. mrigala*.

MATERIALS AND METHODS

Fish

Healthy advanced fingerlings of mrigal carp *(Cirrhinus mrigala)* having an average weight 9 ±1.0g, and total length of 8cm ± 2cm were obtained from Aliyardam, Tamil Nadu. Fishes were transferred to concrete tank and kept for two weeks to acclimatize. After acclimatization, the fish were divided into five groups of 50 specimens in each treatment.

Probiotics and Prebiotics

The lyophilized ample of probiotic bacteria i.e. *Enterococcus faecalis* (Figure 1) were obtained from the Department of Microbiology in PSG- IMSR Coimbatore, Tamil Nadu, India. The sub culture were maintained on Nutrient agar Stored at 37°**C** in the hot air oven for further use, and the prebiotics (dietary carbohydrate) i.e. *Rice bran* were collected from the local market of Coimbatore, Tamil Nadu, India.

Experimental food preparation

For group A; Normal balanced feed composed of 42% fish meal

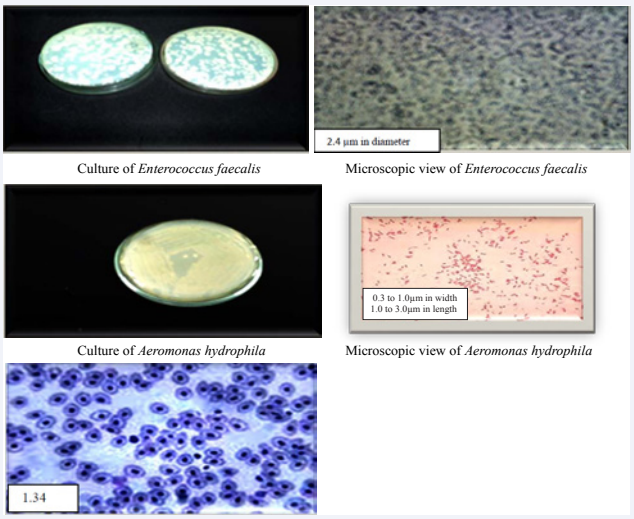

Figure 1 Microscopic view of nucleated fish R. B. C.

and soya been meal, 15% tapioca powder and corn flour, 20% groundnut oil cake, 3% mineral- Vitamin mixture 5% egg white and 15% wheat flour was used as control diet (carbohydrate: 24% protein: 39%: lipid: 11% and ash: 9%).The diet used in the experiment B group were commercial carp food 200gm with 1.5 ml of *E. faecalis* (10^7dil). / day. The diet used in the experiment C group were dietary carbohydrate i.e. *Rice bran*.

Experimental setup

Test group- 1, Fishes was treated with 200 mg of control feed. Test group-2, Fishes was treated with 200 mg feed+1.5 ml of *Enterococcus faecalis* (10^7 dilutions). Test group-3, were treated with 200 mg of Rice bran.

Blood sampling and challenge study

First blood sample collection was done in before challenge study , infectious challenge with *A. hydrophila* was done in the second week last day, second blood sample collection was done in after first challenge study, second infectious challenge with *A. hydrophila* was done on forth week last day, Third blood sample collection was done after second infectious challenge study.

Blood sampling and storage

In all tested group, sample blood was taken though caudal vein for six week in two week intervals. Blood sample was taken for evaluation on the same day. The extra blood was suddenly refrigerated for 12 hrs, then separated and stored at -20˚C until used.

Bacterial strain and challenge study

A virulent strain of *A. hydrophila* (kindly received from Department of Microbiology in PSG-IMSR, Coimbatore, Tamil Nadu, India.) was inoculated in a tryptone soy broth and was incubated at 30°C, after centrifugation at 650 rpm for 15 Min, cells were prepared in PBS. At the end of treatment, twenty five fish in each of the groups were inject intraperitoneally with 0.1 ml of $2\times LD_{50}$ suspension of the bacteria 1.6×10^7 colony cfu / fish in PBS. Daily mortality was recorded for 16 days and the cause of death was ascertained by re isolating the bacteria from the liver and kidney of dead fish [35]. Relative percentage survival (RPS) was calculated as follows RPS (%) = Mortality of untreated control-Mortality of treated / Mortality of untreated control × 100 **Lysozyme activity** was measured by adapting the method described by [36]. The lysozyme activity was expressed as IU ml-1 per mg of serum protein.U/ml = (OD1-OD2/4×0.001×2) × 1000

Serum bactericidal activity was followed by [37].

Total serum protein samples were analyzed for total protein using the method outlined by [38].

Serum globulin content was measured using a standard albumin estimation kit and the globulin content was estimated by subtracting albumin from total protein.

Hematology

Leukocyte count (WBC) where counted by the method of [39] using haemocytometer.

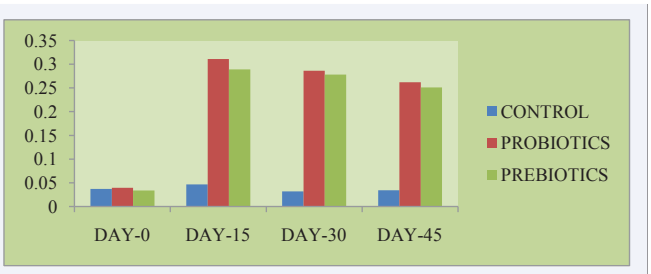

Figure 2 Serum lysozyme count (U/ml^{-1}) of *Cirrhinus mrigala* fingerlings.

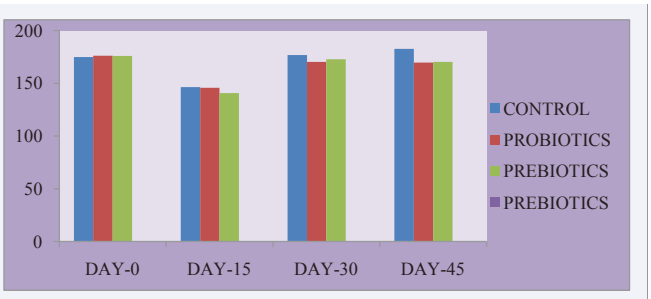

Figure 3 Serum bactericidal activity (%) of *Cirrhinus mrigala* fingerlings.

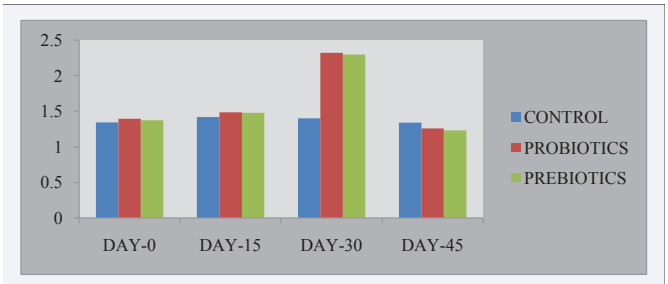

Figure 4 Serum protein level (g/dl) of *Cirrhinus mrigala* fingerlings.

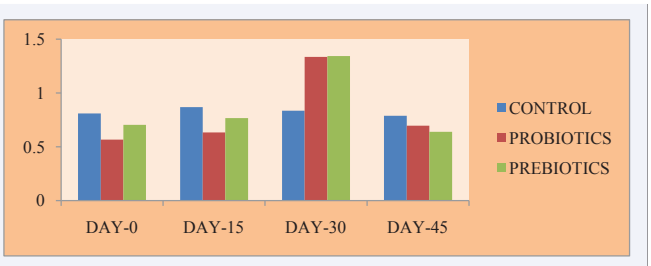

Figure 5 Serum globulin level (g/dl) of *Cirrhinus mrigala* fingerlings.

Red blood cell count (RBC) where determined as described by [40].

Packed corpuscular volume (PCV) was determined by centrifugation at 2000 rpm for 20 min. A suitable quantity of whole blood mixed with an anticoagulant is centrifuged in hematocrit tube until all blood cells are packed at the bottom of the tube. The volume occupied by the packed blood cells gives the PCV or hematocrit value.

Table 1: The effects of probiotics and prebiotics supplemented diets on biochemical and hematological parameters of *C. mrigala* on during 6 week study.

PARAMETERS	GROUPS	DAY-0	DAY-15	DAY-30	DAY-45
Serum lysozyme count(U/ml^{-1})	Control probiotics prebiotics	0.0370 ± 0.0009 0.0394 ± 0.0042* 0.0340 ± 0.0009*b	0.0465±0.0010*a 0.3110±0.0009*a 0.2890±0.0004*a	0.0320±0.0009*a 0.2860±0.0053*a 0.2782±0.0009*a	0.0345±0.0009*a 0.2620±0.0031*a 0.2510±0.0021*a
Serum bactericidal Activity (%)	Control probiotics prebiotics	174.8±24.299 176.2±19.522 175.8±11.203	146.4±8.084* 145.8±7.466* 140.6±7.190*	176.8±17.969* 170.2±12.858* 172.8±9.173*	182.6±15.723* 169.5±7.788* 170.2±12.704*
Serum protein level(g/dl)	Control probiotics prebiotics	1.345±0.023 1.396±0.046* 1.374±0.024	1.420±0.037* 1.486±0.069* 1.478±0.015	1.402±0.021*a 2.320±0.056* 2.296±0.026*	1.340±0.022*a 1.260±0.026*b 1.232±0.028*b
Serum globulin level(g/dl)	Control probiotics prebiotics	0.809±0.045* 0.576±0.018*a 0.704±0.014*a	0.868±0.0283*b 0.634±0.009*a 0.766±0.008*a	0.834±0.019*a 1.334±0.013* 1.342±0.016*a	0.788±0.033*a 0.695±0.005*a 0.638±0.007*a
Leukocyte count(/mm^3)	Control probiotics prebiotics	28.60±0.129 27.24±0.106* 27.84±0.127	31.40±0.091*a 37.54±0.102*a 37.20±0.108*a	32.84±0.120*a 35.44±0.102*a 34.80±0.129*a	30.24±0.102*a 31.84±0.102*a 29.90±0.044*a
Red blood cell count(×10^{6}cells/mm^3)	Control probiotics prebiotics	1.200±0.108*a 1.360±0.021*b 1.348±0.017*b	1.346±0.138* 1.420±0.043* 1.390±0.019*	1.314±0.052*b 1.380±0.012* 1.352±0.010*	1.328±0.020*b 1.402±0.024*b 1.365±0.027*
Packed cell volume(PCV-%)	Control probiotics prebiotics	26.5±1.080* 25±0.912*b 25.4±0.712	26.8±0.678 26.5±0.725 26.8±0.832	26±1.825 25.8±0.726* 25.8±0.648*b	24.8±5.691 26.2±0.832* 26.9±1.003

Table 2: Effect of probiotics and prebiotics on cumulative mortality pattern during 16 days post challenge with *A. hydrophila* in *C. mrigala.*

GROUPS/DAYS	DAY-4	DAY-8	DAY-12	DAY-14-16
Control	5%	15%	20%	25%
Probiotics	5%	10%	15%	10%
Prebiotics	5%	10%	10%	5%

Statistical analysis

Values were presented as (n = 4) arithmetic mean ± standard deviation **(SD).** The data were statistically evaluated by **One-Way** analysis of variance **(ANOVA)** followed by **Post Hoc multiple comparison test** using **SPSS** software. The levels of significance **P < 0.05** was considered as statistically significant and **P < 0.01** as highly significant.

RESULTS

The erythrocyte observed in *C. mrigala* was found to be elongated and nucleated unlike the other vertebrates**.** Similar results were reported [41].

Serum lysozyme count (U/ml^{-1})

The results of the present study revealed that the serum lysozyme activity from the serum of *C. mrigala* was reported highest in probiotics treated group (0.2860 ± 0.00) on 30th day and was observed to be decreased in prebiotics group (Figure 2) (0.2782 ± 0.00) and control group (0.0320 ± 0.00) (Table 1). The Serum lysozyme activity was increased significantly in the fish fed for 15th day with feed supplemented with probiotics and prebiotics **(P <0.05).**

Serum bactericidal activity (%)

Was significantly increased in probiotics and prebiotics treated groups (Figure 3) (Table- 1). Serum bactericidal activity was high in probiotic group when compared with prebiotic and control group, where as the bacterial count was less in probiotic group (169.5 ± 7.788), when compared with prebiotic (170.2 ± 12.70) group, and control group (182.6 ± 15.72) on 45th day of the experiment.

Serum protein level (g/dl)

The results of the investigation reveal that the serum protein content of *C. mrigala* was among the control and experimental groups (Table1). The serum protein was increased significantly in the probiotics group (Figure 4) (2.320 ± 0.00) and prebiotics group (2.296 ± 0.02) on the 30th day **(P <0.05).** The serum protein was high in probiotics group (1.260 ± 0.02) when compared with prebiotics (1.232 ± 0.02) group on 45th day.

Serum globulin level (g/dl)

The results of the investigation reveal that the serum globulin content of *C. mrigala* among the control and experimental groups (Table 1). The serum globulin was `increased significantly in the probiotics (Figure 5) (1.334 ± 0.01) and prebiotics (1.342 ± 0.01) treated group for the 30th day **(P <0.05).** The serum globulin level was high in probiotics (0.695 ± 0.00) group when compared with prebiotics (0.638 ± 0.00) group on 45th day.

Total leukocyte count (/mm^3)

Blood is a very good indicator in determining the health of an organism [42]. The present study indicates that there was a

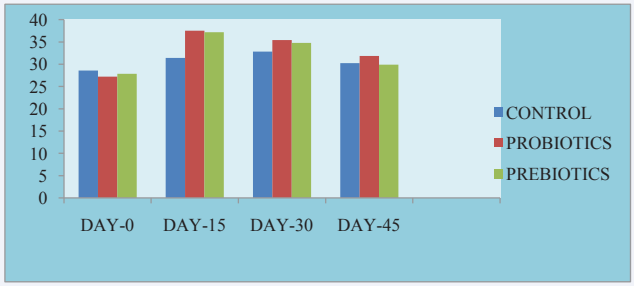

Figure 6 Leukocyte count (/mm^3) of *Cirrhinus mrigala* fingerlings.

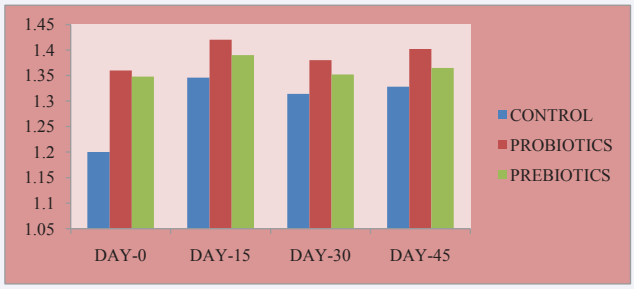

Figure 7 Red blood cell count (×10^{6}cells/mm^3) of *Cirrhinus mrigala* fingerlings.

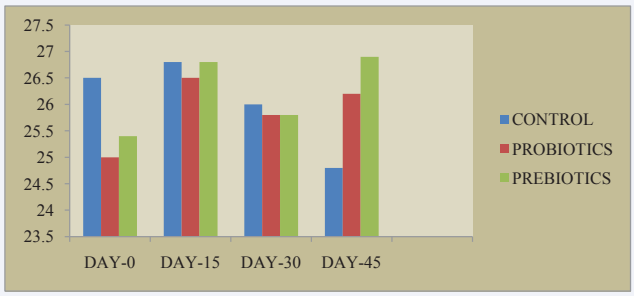

Figure 8 Packed cell volume (PCV-%) of *Cirrhinus mrigala* fingerlings.

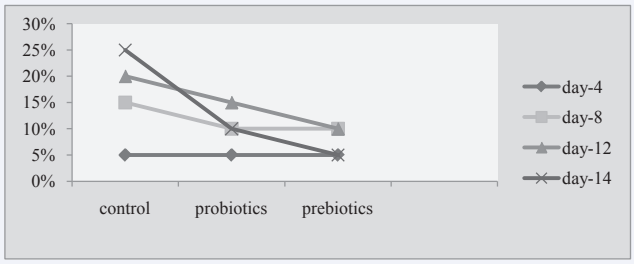

Figure 9 Percentage of mortality (%) of *Cirrhinus mrigala* fingerlings after the challenge study.

significant increase in **TLC** on the 15th day in probiotics (37.54 ± 0.10) and prebiotics (37.20 ± 0.10) treated group (Figure 6) (Table 1). The leukocyte count was also high in probiotics (31.84 ± 0.102) group when compared with prebiotics (29.90 ± 0.04) group and control (30.24 ± 0.10) group on 45th day of experiment.

Red blood cell count (×10^{6}cells/mm^3)

The present study, indicates that there was a significant increase in **RBC** count in both probiotics and prebiotics (1.420 ± 0.04) and (1.390 ± 0.01) treated groups compared with control group on 15th day. The **RBC** count also high in probiotics (1.402 ± 0.02) group when compared with prebiotics (1.365 ± 0.02) and control (1.328 ± 0.02) (Figure 7) group on the 45th day (Table 1).

Packed cell volume (PCV-%)

In this study, the high level of packed cell volume was observed in the probiotics (26.2 ± 0.83) and prebiotics (Figure 8) (26.9 ± 1.00) treated groups on 45th day. The packed cell volume also high in probiotics and prebiotics group when compared with control (24.8 ± 5.69) group on 45th day (Table 1).

Mortality rate (%)

In this study, High level of mortality rate of *C. mrigala* (65%) was observed in control group (Figure 9). The mortality of probiotics and prebiotics group where 40% and 35% respectively (Table 2). After challenge study with *A.hydrophila.*

DISCUSSION

Probiotics are substances or organisms that contribute to the intestinal microbial balance [43], defined probiotics as live microbial feed supplements which exert useful effects on the host animal body by improving its intestinal microbial balance. Research on the benefit of probiotics in aquatic animals has increased the demand for sustainable aquaculture system [44]. Similar studies were administered at Trakia University, Bulgaria, Stara Zagora, with rainbow trout *Oncorhynchus mykiss* with supplementation of prebiotic Bio-Mos.

Lysozyme enzymes also Involves the hydrolyzation of the peptidoglycan layer of bacterial cell walls through cell lysis process. Lysozymes are associated with the defense mechanism against Gram positive bacteria, but also having the ability to destroy Gram-negative bacteria cells as well. Furthermore, this lysozyme is known to trigger an opsonin of the complement system and phagocytic cells [45]. In this study, the serum bactericidal activity increased significantly **($p < 0.05$)** in probiotics and prebiotics treated groups when compared with and control groups**.** However, the presence of anti *A. hydrophila* antibody in the treated fish could be the cause for the increased bactericidal activity. Similar studies were carried out [46,22]. Serum total protein and globulin are considered as better indicators for determining immune system activation [47]. All the immunological active protein of the blood is derived from the gamma globulin fraction. A healthy immune system is maintained by gamma globulins. Serum albumin and globulin values in the fishes treated with various immunostimulants were always higher than control [48]. Increase in the serum protein, globulin and albumin levels is thought to be related with a good innate response in fishes [49]. The increase in serum protein content might be in part due to an increase in the white blood cells, which is a important source of serum protein production such as complement factors, lysozyme, and bactericidal peptides [31].

After the fingerlings were intra peritoneally challenged with *A. hydrophila*. Significant increase in TLC where mainly due to

the immune response of the fish immune system against the bacterial invasion. The gradual reversion of the leukocyte count back to the normal may be indicative of recovery from systemic damage. The present study falls in line with the study of [20], who observed that significant increase of the white blood cells count was not observed in all the treatments except for chitin fed fish. The WBCs afford protection against infectious pathogen caused by microbial and chemical factors. In this study, administration of probiotics and prebiotics in treated groups enhanced the survival rate after a challenge with live *A. hydrophila.* The highest survival rate (60%, 65%) was observed in the probiotics and prebiotics treated groups respectively. The present findings are inagreement with the results [50].

CONCLUSION

The present results showed that the oral administration of probiotics and prebiotics can enhance the specific and non-specific immune responses. This appears to be achieved primarily by increasing lysozyme activity, serum bactericidal power, serum protein and globulin levels. Hematological parameters such as WBC, RBC, PCV- values. Furthermore, the data reported in this study shows that a 0.5% / kg probiotics and prebiotics supplementation can increase the resistance to *A. hydrophila* and reduce mortality rate in *C. Mrigala.*

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