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Effect of Probiotic on Microbiological and Haematological Responsiveness of Cat fish (*Heteropnuestes fossilis*) Challenged with Bacteria *Aeromonas hydrophila* and Fungi *Aphanomyces invadans*

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Abstract

The use of probiotic for disease prevention and improved nutrition in aquaculture is becoming popular due to an increasing demand for environment friendly aquaculture. Here we used *Bacillus subtilis* as a probiotic to fish to evaluate the effect of probiotic on microbiological and haematological responsiveness of cat fish (*Heteropnuestes fossilis*) challenged with bacteria *Aeromonas hydrophila* and fungi A*phanomyces invadans*. *Heteropneustes fossilis* were collected from local market at Tirunelveli, Tamil Nadu, India. Fish were subjected into microbiological, haematological, physiological observation. In *H. fosilis*, probiotic accepted fishes gained more weight than that of the control fishes fed with control diet. The gut micro flora of *H. fossilis* was found to be 6.3×10^6, 5.7×10^7, 5.4×10^5 and 5.1×10^5 cells in D1 treatment fishes on 10^4,10^5,10^6 and 10^7 dilutions respectively. The microbiological estimation also showed a dual increase in trial count in T2 injected fish than that of the T3 injected fish. Many factors can influence the immune response of fish. Among them are stressors and environmental factors are natural. In the present investigation behavioural symptoms to pathogenicity such as imbalance, restlessness and avoidance of food were observed. Pathological symptoms include fin necrosis and tail rot which were also observed. In some cases septicaemic ulceration was noticed. Haematological parameters elicited changes which are able to reveal some clues for diagnosis and prognosis of the disease state. T2 fishes were inflicted alterations in TEC, TLC, DLC, and Hb content which indicated decrease state of immunity, when compared with T3 fishes. Bacteria injected fishes showed good healthy status whereas fungal injected fishes showed non healthy status of fish.

Keywords: Probiotic; Microbiological; Haematological; Cat fish; Bacteria; Fungi

Introduction

Only in the last few years, aquaculture has undergone rapid advancement. The principle reasons for the increased interest and development of fish farming are due to the recent advances in the development of fish culture techniques in the world particularly in the field of husbandry and management of culture system and the development of standardized artificial breeding technologies such that supply of seeds is guaranteed and controlled by the fish farmer. A large number of reports are available on general biology in relation to food, feeding habits and breeding [1]. However, information in relation to nutrient requirements of the fish protein is limited. Development of economical feed mixture is an important factor in fish culture in which the growth of fish is influenced by the quality and quantity of the diet. Reports are available on the growth of cultivable fishes using animal and plant sources of protein diets [2]. For optimum fish growth the use of fish meal (25-65%) as higher dietary protein in fish feeds causes more expensive. Hence, the development of low cost and nutritionally balanced diet is in urgent need. The alternative sources of protein either by partial or complete replacement of the fish meal have been studied using various ingredients [3]. This study focussed on investigation attempts have been made to produce the fish feed using fish meal like anchovy, jawala and flour like soy flour, tapioca flour and wheat flour and sunflower oil, aquasavour, vitamin C.

Infectious diseases are considered as one of the main barriers to the successful development and continuation of molluscan and shrimp aquaculture as they limit production in terms of quality, quantity and regularity [4]. Although disease control is an inherent component of any intensive animal production system, controlling disease in the aquatic environment is further complicated by the intimate relationship that exists between pathogens and their host and the frequent use of open production system [5]. However, excessive antimicrobial use can lead to the emergence of bacterial resistance [6,7]. Hence the use of probiotics for disease prevention and improved nutrition in aquaculture is becoming popular due to an increasing demand for environment friendly aquaculture [6]. Probiotics act as growth promoter and reduce the substrate of pathogenic microbes. Commonly available probiotics are *Lactobacillus acidophilus, Bacillus subtilis* and EfinolG (mixture of microbes). Several studies have shown that probiotics improves the growth rate of fishes by improving the immune status of fishes [1,4,8-10]. The use of Probiotics to displace pathogenic bacteria by competitive process is a better remedy than administering antibiotics. *Pseudomonas fluorescens* (AH2) was shown to be strongly inhibitory against *Vibrio anguillarum* and it reduced the mortality rate of rainbow trout injected by *Vibrio anguillarum* [5]. Improved disease resistance has also been observed in cod fry fed with dry feed containing *Carnobacterium divergens* [11,12] showed that the survival and growth of the black tiger shrimp (*Penaeus monodom*) [12]. Thus, probiotics have been shown to be effective in a wide range of

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species for the promotion of growth, enhanced nutrition, immunity and survival.

Materials and Methods

Heteropneustes fossilis were collected from local market in Tirunelveli, TamilNadu, India and transported to CARE aqua farm. They were acclimatized to laboratory condition for a week. Ambient temperature 29±1˚C and pH 7, 1±0.5mg/lr, was maintained, throughout the experiment and 1/3rd of water was renewed daily.

Feed formulation

Fishes were fed regularly with an artificial balanced diet made up of wheat flour, tapioca flour, soya flour, vegetable oil, anchovy, jawala to control fishes and *Bacillus subtilis* was added in the diet to experimental fishes (*B. Subtilis* was purchased from Xi'an Lyphar Biotech co., Ltd, China). Fishes were reared in plastic troughs (capacity 40 litres). The following ingredients were used to prepare feed pellet for *Heteropneustes fossilis* for 100 mg fish feed.

Anchovy	-	26.9 gm
Soy bean	-	25 gm
Jawala Acetes	-	20 gm
Tapioca meal	-	10.9 gm
Wheat flour	-	10 gm
Sunflower oil	-	5.8ml
Mono sodium phosphate	-	0.5 gm
Aquasavour	-	0.3 gm
Ascorbic acid	-	0.02 gm
Probiotic concentration	-	2 mg

Fish meal

Anchovy and Jawala prawn were purchased from local fish market. They were powdered and sieved to required size. The protein content of the fish meal was 60%.

Flour: Wheat, soybean and tapioca flour were used. Tapioca flour acts as a binder and source of carbohydrate. Gelatinization of tapioca flour improves the stability of the feed.

Oil: 5.8 ml of sunflower oil was used in the food formulation.

Constituents

	Energy	-	884 cal
	Total fatty acids	-	100 gm
i.	SFA	-	12 gm
ii.	MUFA	-	28 gm
iii.	PUFA	-	60 gm
iv.	Trans fatty acids	-	BDL

i. Saturated Fatty Acid
ii. Mono Saturated Fatty Acid
iii. Poly Saturated Fatty Acid
iv. Below Detectable limit (0.05%)

Vitamin: 0.02 gm Vitamin C was used to formulate fish feed.

Feed preparation

All powdered ingredients were weighed separately and mixed with required amount of hot water to make semi moist dough and sterilized .Then 5.8ml sunflower oil was added under appropriate temperature and the probiotic *B. subtilis was* also added under aseptic condition. This mixture was pelleted and dried.

Experimental design

The experimental fishes *H. fossilis* were reared in plastic trough of 40lr capacity. Twelve troughs containing six fishes in each were taken and filled with water. This was divided into two groups as Diet 1 (D_1) treatment (4 troughs) and Diet 2 (D_2) probiotic treatment (8 troughs). The control fishes were fed with control basal diet while the probiotic treatment fishes were fed with *B. subtilis* added diet for 21 days feeding trial. Before the feeding trial the gut micro flora and the haematology of the experimental fishes were analysed.One third of water was changed daily. After completion of feeding trial the fishes were divided into three treatments (D_1) group fishes was kept as treatment 1 (T1) The D_2 group was further divided into two treatments with four troughs of fish for each. The treatment II fishes were injected *A. hydrophila* in treatment III were *A. invadans* injected fishes. This disease challenge was done for 15 days and the microbiological and haematological changes were observed and recorded.

LD_{50}

The LD_{50} *Aeromonas hydrophila* (10^6 dilution) by one fish/dose, six fishes each replicate, were injected intramuscularly, in 0.1 ml concentration. *Aphanomyces invadans* (10^5 dilution) by one fish/dose, six fishes each replicate, were injected intramuscularly, in 0.1 ml concentration.

Disease challenge

Before the treatment the control fishes were fed with control feed and the experimental fishes were fed with probiotic containing feed. After the treatment all the experimental fishes were fed with control feed including control fishes. The treatment (T1) fishes were injected with physiological saline. The treatment II (T2) and treatment III (T3) fishes were injected with 0.1 ml of *A. hydrophila* and *A. invadans* respectively.

Disease challenge was done on 31st day by intramuscular injection of *A. hydrophila* and *A. Invadans* to (T2) and (T3) group fishes respectively. At the start of experiment (T=0) fishes in (T2) and (T3) of the fishes were fed with feed containing probiotic, while the remaining 1st group was fed with non probiotic feed. The immunological parameters (RBC count, WBC count, DLC, PCV, MCV, MCHC, Haemoglobin content of blood) of the fish were recorded on (31, 30, 35 and 40 days.) in all the three treatments.

Microbiological estimation

The samplings were made on 0, 30, 35, 40and45 days for microbial investigation of gut of experimental fish *H. fossilis* in order to check the changes in gut micro flora after probiotic feeding.

Enumeration of total heterotrophic bacterial count

The experimental fish *H. fossilis* were taken and wiped with alcohol to remove the surface bacteria. The fish were dissected and the intestine was removed and homogenized with sterile distilled water and grinded with morter and pestle. 1ml of homogenized solution was taken and added into sterile blank water representing as 10^{-1} dilution. Then it was serially diluted upto 10^{-10} dilution. For Heterotrophic bacterial count 0.1 ml was taken from 10^{-4} to 10^{-7} dilution and spread plated into sterile nutrient agar plates (10^{-4} to 10^{-7}). Duplicates were maintained for each dilution. The plates were incubated at 37˚C for 24 hrs. After incubation colonies were observed and recorded.

Collection of blood sample

Blood was obtained by puncturing the heart by using 1ml insulin syringe. Before that the syringe and blood collecting vials are coated with anti-coagulant heparin. Anticoagulated blood was used for the analysis of the blood variables except differential leucocyte count and serological parameter.

Total erythrocyte count

Enumeration of total erythrocytes was done with Haemocytometer. Blood was diluted 200 times in the standard RBC pipette with RBC diluting fluid.

Total leukocyte count

Enumeration of total leucocytes was done using Haemocytometer.

Differential leucocyte count

For differential leucocyte count minimum of 100 leucocytes were classified and counted from each smear. They were indentified under 40X magnification.

Haemoglobin content

Sahlis's method had been employed for estimating haemoglobin content of blood.

Mean Corpuscular Haemoglobin (MCH)

It's used to determine the average haemoglobin content in single red cell in micro gram.

Haematocrit Value (Packed Cell Volume-PCV)

The haematocrit value (Hk) determines the ratio of the volume of the blood cells to that of blood plasma. The haematocrit value (Hk) is expressed as the percentage fraction of blood cells, in the total volume.

Mean Corpuscular Volume

The average volume of a single red cell in cubic microns.

Mean Corpuscular Haemoglobin Concentration (MCHC)

To determine the haemoglobin content of 20 µm of the packed cells as percentage as opposed to the percentage of haemoglobin of whole blood.

Results

The experimental fish *H. fossilis* readily accepted the probiotic diet *(B. subtilis)* and basal diet (control). No mortality occurred during the feeding trial. Length and weight gain of *H. fossilis* fed with probiotic diet was higher than the fishes fed with control diet. The gut micro flora of *H. fossilis* was found to be 6.3×10^6,5.7×10^7,5.4×10^5 and 5.1×10^5 cells in D1 treatment fishes on 10^4,10^5,10^6 and 10^7 dilutions respectively (Table 1) on initial day (day 0). On 30th day the gut micro flora of *H. fossilis* fed with control diet and probiotic diet was found to be 5.7×10^7, 4.8×10^7, 5.1×10^6 and 4.3×10^5 cells on 10^4,10^5,$10^6$and 10^7 dilutions respectively (Table 2). On day 35, 40 and 45 the microbial analysis was done in three treatments such as T1, T2 and T3. The gut micro flora of T2 fishes was found to be 7.4×10^6, 8.1×10^5, 7.9×10^5 and 4.7×10^5 on 35th day in 10^4,10^5,10^6 and 10^7 dilutions respectively. This showed a gradual decrease in T3 fishes (Table 3). On 40th day its showed gradual decrease of 7.2×10^6,7.3×10^5,6.9×10^5 and 4.3×10^5 in 10^4,10^5,10^6 and 10^7dilutions respectively. But in T3 fishes showed fluctuation (Table 4) when compared to T3 fishes on day 35.

The fungal and bacterial load in T2 and T3 fishes was decreased on day 45 (Table 5) when compared to days 40. The fungal colonies showed only a slight limitation in the growth recording 4.1×10^6, 5.7×10^5, 6.2×10^5 and 4.1×10^5 on day 45 in 10^4 ,10^5 ,10^6 and 10^7 dilutions respectively. The bacterial load and fungal load were found to be least in T2 and T3 on day 45. The results of gut microflora were showed in Tables 1-5.

Intramuscular injection of *Aeromonas hydrophila* and *Aphanomyces invadans* showed slight to severe dermomuscular lesions, in the experimental fish *H. fossilis*. Among the three treatments external lesion initially developed as a blanched area with superficially abrading lesion and excavated lesion with swelling in both probiotic treated (T2 and T3) fishes. But in the case of probiotic fed *A. hydrophila* injected (T2) group the infections gradually decreased during the experimental period from day 9(+++) until day 15(+). Whereas in *A. invadans* injected group (T3) lesion slightly decreased on day 9(+++) and further decreased on day 15(++). In the *A. hydrophila* injected group external lesion was reduced in the probiotic feeding than *A. invadans* injected group. The control fish did not develop any lesion.

The values of various Haematological indices of T1,T2,T3 are shown in Tables 6-13 i.e, TLC, TEC, DLC, PCV, Hb content, MCH, MCV, MCHC respectively.

TLC of T1 did not show much variation and found to be 3.3 (Cells/mm^3) on 30th day and 3.4 (Cells/mm^3), 3.1 (Cells/mm^3), 3.0 (Cells/mm^3) on day 35,40,45 respectively .The TLC of *A. hydrophila* (T2) injected fishes showed a decrease on the day 35 and gradually increased on day 45. In *A. invadans* (T3) injected fishes too the leucocytes showed a decrease on 35th day 2.6 (Cells/mm^3) and a slight increase on day 45th day 4.0 (Cells/mm^3) were observed (Table 6).

The TEC in T1 fishes were found to be normal from the 30th day 3215 (Cells/mm^3) to 45th day 3100(Cells/mm^3). In T2 fishes the red blood cells showed increased level on day 35 and it gradually decreased on day 45. In case of T3 also red blood cells increased on day 35 and slightly decreased on day 45 (Table 7).

DLC in T1 fishes were found to be from the 30thday to 45th day showed fluctuation in T2 fishes when compared with T1 fishes (Table

Dilution Factor	D1	D2
10^4	6.3×10^6	5.4×10^6
10^5	5.7×10^7	5.2×10^6
10^6	5.4×10^5	5.0×10^5
10^7	5.1×10^5	5.1×10^4

Table 1: Heterotrophic Bacterial Count (Day 0).

Dilution Factor	Colonies in number
10^4	5.7×10^7
10^5	4.8×10^7
10^6	5.1×10^6
10^7	4.3×10^5

Table 2: Heterotrophic Bacterial Count (Day 30).

Dilution Factor	T1	T2	
104	6.7 × 106	7.4×106	4.1 ×105
105	6.1 × 106	8.1 × 105	4.0 ×105
106	8.3 × 105	7.9 × 105	5.1 ×104
107	8.0 × 105	4.7 ×105	3.2 ×104

Table 3: Heterotrophic Bacterial Count (Day 35).

Dilution Factor	T1	T2	T3
104	6.4 × 106	7.2×106	4.3 ×105
105	6.2 × 106	7.3 × 105	4.0 ×105
106	5.8 × 106	6.9 × 105	7.9 ×104
107	7.3 × 105	4.3 ×105	5.7 ×104

Table 4: Heterotrophic Bacterial Count (Day 40).

Dilution Factor	T1	T2	T3
104	6.7 × 106	4.1×106	4.0 ×105
105	6.3 × 106	5.7 × 105	7.9 ×104
106	7.1× 105	6.2 × 105	4.1 ×104
107	5.8 × 105	4.1 ×105	3.2 ×105

Table 5: Heterotrophic Bacterial Count (Day 45).

Day	Sample	Mean	SD
30th Day	T1	3.3	1.2
	T2	4.1	1.7
	T3	3.9	1.5
35th Day	T1	3.4	1.3
	T2	3.9	0.9
	T3	2.6	0.7
40th Day	T1	3.1	0.8
	T2	3.8	1.3
	T3	3.1	0.8
45th Day	T1	3.0	1.2
	T2	4.3	1.4
	T3	4.0	0.7

Table 6: Comparison of TLC (Cells/mm3) in *H. f ossilis* administered w ith *A. hydrophila* and *A. invadans.*

Day	Sample	Mean	SD
30thDay	T1	3.2	0.7
	T2	3.2	1.5
	T3	2.8	1.0
35th Day	T1	3.1	0.8
	T2	3.5	1.2
	T3	3.6	1.0
40th Day	T1	3.4	0.5
	T2	3.7	1.7
	T3	3.7	1.3
45th Day	T1	3.1	0.2
	T2	3.7	1.4
	T3	3.9	0.9

Table 7: Comparison of TEC(Cells/mm3) in *H. f ossilis* administered w ith *A. hydrophila* and *A. invadans.*

Cells	Duration							
	Bacteria Injected							
	Control (T1)				Test (T2)			
	30th day	35th day	40th day	45th day	30th day	35th day	40th day	45th day
Neutrophils %	60	40	40	39	60	50	56	42
Eosinophils %	2	8	10	10	5	5	5	9
Basophils %	6	12	10	9	3	2	1	1
Lymphocytes %	30	40	39	41	32	39	38	48
Monocytes %	2	0	1	1	0	4	0	0

Table 8: DLC(%) in *H. f ossilis* administered w ith *A. hydrophilia* (106 CFU/0.1ml)

Cells	Duration							
	Fungus Injected							
	Control (T1)				Test(T3)			
	30th day	35th day	40th day	45th day	30th day	35th day	40th day	45th day
Neutrophiles %	52	40	45	48	43	62	51	50
Eosinophiles %	3	8	10	9	5	3	4	5
Basophiles %	5	10	5	6	10	2	1	1
Lymphocytes %	33	40	40	37	42	30	41	44
Monocytes %	7	2	0	0	0	3	3	0

Table 8.1 DLC(%) in *H. f ossilis* administered w ith *A. invadans* (105 CFU/0.1 ml)

Duration	Bacteria Administered				Fungus Administered	
	Control (T1)		Test (T2)		Test (T3)	
	PCV%	Plasma	PCV%	Plasma	PCV%	Plasma
30th day	25	75	25	75	26	74
35th day	25	75	22	78	14.7	85.3
40th day	24	76	20	80	13.2	86.8
45th day	25	75	18.2	81.8	11.1	88.1

Table 9: Haemetocrit value of *H. f ossilis* administered w ith *A. hydrophilia* (106 CFU/0.1ml) and *A. invadans* (105 CFU/0.1 ml).

Duration	Bacteria Injected		Fungus Injected
	Control (T1)	Test (T2)	Test (T3)
30th day	19.8	19.7	20
35th day	19.7	14.5	15
40th day	19.8	10	13
45th day	19	11.1	10

Table 10: Comparison of Haemoglobin content (in gram) of blood *in H. f ossilis* administered w ith *A. hydrophilia* (106 CFU/0.1ml) and *A. invadans* (105 CFU/0.1ml)

Duration	Bacteria Injected		Fungus Injected
	Control (T1)	Test (T2)	Test (T3)
30th day	0.0049	0.0047	0.0050
35th day	0.0051	0.0037	0.0054
40th day	0.0050	0.0034	0.0050
45th day	0.0064	0.0044	0.0045

Table 11: Comparision of MCH (in microgram) *in H. f ossilis* administered w ith *A. hydrophilia* (106 CFU/0.1ml) and *A. invadans* (105 CFU/0.1ml).

Duration	Bacteria Injected		Fungus Injected
	Control (T1)	Test (T2)	Test (T3)
30th day	0.00622	0.00622	0.00652
35th day	0.00638	0.00556	0.00525
40th day	0.00600	0.00671	0.00510
45th day	0.00801	0.00723	0.00501

Table 12: Comparision of MCV (in cubicmicrons) *in Heteropnuestes f ossilis* administered w ith *Aeromonas hydrophilia* (106 CFU/0.1ml) and *Aphanomyces invadans* (106 CFU/0.1ml).

8). In T3 fishes also showed a decrease level of DLC from 30th day to 45th day (Table 8.1).

The PCV and total plasma level was found to be higher in T2 fishes were higher than compared to T1 and T3 fishes. Total plasma increased from 30th day to 45th day in T3 (Table 10) when compared to T1 and T2 (Table 9).

Haemoglobin content of T3 fishes were higher than T1 and T2 (Table 10). The MCH level of T3 was higher than that of the T1 and T2

Duration	Bacteria Injected		Fungus Injected
	Control (T1)	Test (T2)	Test (T3)
30th day	79.2	78.8	76.92
35th day	78.80	65.90	102.04
40th day	82.50	50.00	98.48
45th day	76.00	60.98	90.09

Table 13: Comparision of MCHC (%) *in Heteropnuestes f ossilis* administered w ith *Aeromonas hydrophilia* (106 CFU/0.1ml) and *Aphanomyces invadans* (105 CFU/0.1 ml).

(Table 11). The MCV level showed fluctuations (Table 12); MCHC level of T3 showed higher than that of T1 and T2 (Table 13). No mortality was observed in T1, whereas in T2 2% and T3 6% were noticed. Lesions induced by *A. invadans* were higher than *A. hydrophila* on *H. fossilis* (Table 14).Because of probiotic feeding *H. fossilis* gained weight in D1 was about 10.25 gm, and in D2 about 8.0 gm (Table 15).

Discussion

The recent intensification of aquaculture has lead to a number of fish diseases due to environmental and physiological stress in fish population. Overcrowding, handlings of fish and water quality have resulted in disease outbreaks. The present investigation reports a study of microbiological and haematological responsiveness of *H. fossils* fed with probiotic diet during experimental infection by A. *hydrophila* and *A. invadans*. This study reports *A. hydrophila* and *A. invadans* caused infection and severe lesions were noticed in T2 and T3.

A. hydrophila is disseminated as a cosmopolitan especially in aquatic environments which provides ample opportunity to fishes and leads to infection and then causing mortality [13]. So, the pathological symptoms made by *A. hydrophila* in this study, were similar to the studies made earlier [14].

TEC in *A. hydrophila* infected *H. fossilis* and *A. invadans* infected *H. fossilis* exhibited a significant decrease on prolonged exposure. Previous studies reported a hike in TEC during the unhealthy state of fish [13,15]. Accordingly, high counts are associated with the abnormal conditions of fish. Hence, a sudden increase in the TEC is indication of a severe infection by the opportunistic bacteria and fungi. This might have been accomplished by a rapid mobilization of RBC from the haemopoietic tissue, which may transport higher amounts of Oxygen particularly to withstand stress factor caused by *A. hydrophila*[1,16].

TLC also showed an increasing trend in the infected the *H. fossilis* with *A. hydrophila* and *A. invadans***.** Increase in the number of WBC's has been reported by a several investigators. The results of the present study agrees with the works of Innocent research group [17]. DLC studies showed that lymphocytes constituted maximum percentage followed by neutrophils, Ainsworth has suggested that neutrophils and macrophages are responsible for bacterial uptake [18]. Similar observation of neutrophilia an inflammatory response was record by Innocent et al., [19] in *M. montanus* infected by *A. Hydrophila* [19].

Varieties of leukocyte types are involved in innate cellular defense of fish including macrophages, granulocytes and non-specific cytotoxic cells [20]. The above findings support the present study. Monocytes and macrophages are probably the single most important cell mediated immune reponse of fish [21]. They are the primary cells involved in phagocytosis and the killing of pathogens upon first recognition and subsequent infection [22]. Secombes has reported that in fish granulocytes especially neutrophils are the primary cells involved in the initial stages by inflammation. Granulocytes are highly motile, phagocytic and produce reactive oxygen species. The results of the present studies also show significant increase in neutrophils and lymphocytes. Lymphocyte numbers decreased significantly in the post challenge sample while neutrophils and monocyte remain unchanged. Hb content showed a decrease in *H. fossilis* experimentally infected by *A. Hydrophila* leading to anaemia.

The administration of probitics feed in experimentally infected *H. fossilis* elicited alteration in haematological parameters such as TEC, TLC, DLC, MCV, MCH, MCHC, PCV. An analysis of results of present study reveals that *B. sublilis* plays a vital role in increasing length and weight of fish. And also, it did not cause any changes in gut micro flora.

A. hydrophila and *A. Invadans* are potentially effective to the *H. fossilis* in 10^{-4} CFU/ml concentration. The challenging of *A. hydrophila* and *A. invadans* in *H. fossilis*, in terms of immunological responsiveness shows good healthy status. Post challenging of these pathogen causes disease in fishes. But *B. subtilis* is not having any vital role in immune mechanism of fish after post challenging.

Lesions developed by *A. invadans* were higher than that of the *A. hydrophila* induced lesions. So, the probiotic was not efficient in fungal infection. During probiotic feeding weight was also gained when compared to control. So probiotic acts as a one of the growth promoter in *H. fossilis.*

Conclusion

In *H. fosilis*, probiotic accepted fishes gained more weight than that of the control fishes. The microbiological estimation also showed a dual increase in trial count in T2 injected fish than that of the T3 injected fish. Many factors can influence the immune response of fish. Among them are stressors and environmental factors are natural. In the present investigation behavioural symptoms to pathogenicity such as imbalance, restlessness and avoidance of food were observed. Pathological symptoms include fin necrosis and tail rot which were also observed. In some cases septicaemic ulceration was noticed. Haematological parameters elicited changes which are able to reveal some clues for diagnosis and prognosis of the disease state. T2 fishes were inflicted alterations in TEC, TLC, DLC, and Hb content which indicated decrease state of immunity, when compared with T3 fishes. Bacteria injected fishes showed good healthy status whereas fungal injected fishes showed non healthy status of fish.

	30th Day	33rd Day	39th Day	42th Day	45th Day
T1	-	-	-	-	-
T2	-	+	++	++++	+++
T3	-	++	+++	+++	++++

\- Normal Intact skin
-/+ Intact but melanized skin at injection site
\+ Blanching Slight sw elling of injection site
++ Blanching furuncle – like lesion w ith dermal erosion w ith or w ithout hemorrhagic periphery
+++ Extensive blanching lesion w ith furuncle like ulcerated core
++++ Ulcerated lesion w ith underlying necrotic musculature

Table 14: Lesions induced by *A. hydrophila and A. invadans* on *H.Fossilis.*

Diet	Initial weight	Final weight	WG
D1	27.25	37.50	10.25
D2	25.08	33.08	8.0

Table 15: During probiotic feeding Weight Gained (WG).

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