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Protective effect of probiotic diets on haematobiochemical and histopathology changes of *Mystus montanus* (Jerdon 1849) against *Aeromonas hydrophila*

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PEER REVIEW

Peer reviewer

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Comments

This is a well written study about the disease challenge of fish treated with probiotic diet. The results are clear and it is concluded that infected groups of fish maintained on probiotic diets yielded significantly better growth, haematology parameters and histopathology than the control. This article is very useful for fish diet against *A. hydrophila*.
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ABSTRACT

Objectives: To evaluate the protective effect of probiotic diets on haemotobiochemical and histopathology changes of *Mystus montanus* against *Aeromonas hydrophila* (*A. hydrophila*).
Methods: Three experimental groups of fish were fed with a diet supplemented with *Lactobacillus acidophilus* (*L. acidophilus*) (Sporolac), comprising about 0.1 g, 0.2 g and 0.3 g. Control group of fish were fed without *L. acidophilus*. After 60 d of feeding the fishes in experimental group were injected with 1 mL of *A. hydrophila* and were supplemented with probiotic diets. The control group fishes were injected with 1 mL of physiological saline solution alone.
Results: Blood samples were collected for haematobiochemical analysis, while samples of the liver, and gills were examined for path histology after 7 d of infection. The result showed that the growth parameters, weight gain, specific growth rate were better in infected group maintained on the probiotic diet compared to those in control group. The haematology parameters, erythrocyte sedimentation rate, red blood cell, white blood cell, total serum protein, Mg^{2+}, Ca^{2+}, Cl, glucose, cholesterol and total immunoglobulin concentration and the pathohistology of the liver, gills were better in the infected fish maintained on the probiotic diet than those in the group fed the control diet.
Conclusions: The result of the present study showed that *L. acidophilus* is useful as a probiotic agent in *Mystus montanus* against *A. hydrophila*.

KEYWORDS

Mystus montanus, *Aeromonas hydrophila*, *Lactobacillus acidophilus*, Haemotobiochemical, Histopathology

1. Introduction

Fish disease is widely distributed worldwide and is considered to be serious problems in aquaculture[1]. Aquaculture has made significant advances in recent years in the production of a wide range of aquatic organisms, both for human consumption and as ornamental species[2]. Bacterial infections are one of the most important causes of disease problems in Indian aquaculture especially in the production of cat fish[3]. *Aeromonas hydrophila* (*A. hydrophila*) is the most common pathogen, and it can easily spread through accidental abrasions[4]. Among the common bacteria pathogens, *Staphylococcus xylosus*, *A. hydrophila* and *Streptococcus agalactiae* are known to seriously infect

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fish in aquaculture systems, sometimes causing heavy mortalities[5]. The blood parameters of fish provide accurate indications of any changes occurring in the organism as a result of injuries to organs or tissues related to infectious disease, similar to those of warm blooded animals[5,6].

It was reported that the haematological and biochemical parameters of fish can be used to evaluate the health condition of the organism. The microorganisms used as probiotics, including *Lactobacillus*, *Bacillius* and yeasts, have been reported in penaeids and fish[7,8].Probiotics play important roles as immunostimulants and antimicrobial agents. Probiotics are live microbial or cultured product feed supplements that beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating immune function, and improving microbial balance[9]. In aquaculture, probiotics have been used to control diseases, enhance specific and non-specific immunity, provide nutrients and enzymatic functions and improve water quality[9,10]. Especially lactic acid bacteria have been identified to posses probiotic properties and some beneficial advantages, as it enhances the digestive process in its host and would therefore be useful due to its consumers[11]. The fresh water cat fish *Mystus montanus* (*M. montanus*) for about 10% of commercial landings of air-breathing fish in Tamilnadu is one of the highest priced fish due to its tender flesh, few bones, and good taste. It is endowed with remarkable powers of respiration, which enables it to lead an amphibious life and to survive in adverse conditions in swampy areas. It is endemic to catchments of the Tambaraparani River, Tirunelveli district (Tamilnadu, India). In this study, the protective effect of probiotic diets on haemotobiochemical and histopathological changes of *M. montanus* against *A. hydrophila* was evaluated.

2. Materials and methods

2.1. Experimental design

Healthy *M. montanus* were transported from Kumar private fish farm, Tirunelveli and where acclimatized in two concrete tanks measuring (400 cm×150 cm×100 cm). The basal diet were fed three times daily for two weeks and then fish were distributed into 12 plastic containers. All *M. montanus* had similar initial weight (3.46±0.02) g. The experimental protocol included four treatment groups and each treatment had three replicates, and culture period was 60 d. All fishes were fed daily at 9.00 am and 4.00 pm each day. Any remaining diets were collected by siphoning before feeding. Every third day each plastic container was cleaned and the water changed. For water quality control, temperature, pH and dissolved oxygen were measured daily, the level of dissolved oxygen was observed above 7 per mg.

2.2. Diet preparation

The feed was prepared using common ingredients such as wheat flour, fishmeal, soy flour, tapioca flour, multivitamin tablet and cod liver oil which were procured from the local markets at Thoothukudi, Tamilnadu, India. The above ingredients were mixed and grained to make powder form. The food powder is mixed with boiled water to make paste or semi moist dough. The dough was kept in air tight plastic containers for further use. The prepared feed was fed at rate of 5% body weight for twice a day. The ingredients and percentage composition of the basal diet used in the experiment are given in Table 1. In Treatment 1 (T_1), experimental diet consisted of 0.1 g of *Lactobacillus* (Sporolac) was added to the basal diet in each of three plastic containers. In Treatment 2 (T_2), experimental diet of 0.2 g of lactobacilli bacteria (Sporolac) was added to basal diet in each of three plastic containers. In Treatment 3 (T_3), experimental diet consisted of 0.3 g of lactobacilli (Sporolac) was added to the basal diet in each of three plastic containers. The control diet was not added any *Lactobacillius*. The water quality parameters were monitored weekly and the mean values recorded were as follows: temperature (29.47±0.50) °C and pH (7.01±0.11). At the end of the experimental period, three fish from each aquarium were randomly removed for haemotobiochemical and patho-histology analysis.

Table 1

Formulation of basal diet.

Ingredients	Control	T_1	T_2	T_3
Anchovy fish meal	45.0	45.0	45.0	45.0
Wheat flour	20.0	20.0	20.0	20.0
Soy flour	24.0	24.0	24.0	24.0
Tapica flour	9.0	8.9	8.8	8.7
Vitamin and mineral pre mix tablets	1.0	1.0	1.0	1.0
Cod liver oil	1.0	1.0	1.0	1.0
Lactobacillus acidophilus (g)	0.0	0.1	0.2	0.3

T_1: Treatment 1, T_2: Treatment 2, T_3: Treatment 3.

2.3. Growth parameters and rate of feed intake

The growth parameters and rate of feed intake were calculated as previous report[12]. The fish in each treatment were counted and weighed at the termination of the experiment. The growth parameters and feed utilization were calculated as follows:

Weight gain=Final weight of fish-Initial weight of fish

Specific growth rate (SGR)=$100\times(W_2-W_1)/T$

Where, W_1 and W_2 are the initial and final weights and T is the number of days of feeding.

Feed conversion ratio (FCR)=$F_1\times(B_2+B_{dead}-B_1)-1$

Where F_1, B_1 and B_2 are the feed intake, the biomass at the start and end of the experiment and B_{dead} is the biomass of

the dead fish.

Survival rate=final No. of fish/initial No. of fish×100

2.4. Haemotobiochemical analysis

At the end of experiment, blood samples were taken from the caudal vein of an anaesthetized fish by sterile syringe using EDTA solution as an anticoagulant. The blood samples were used for determining erythrocyte count and haemoglobin content[13,14]. Haematocrit value was calculated according to the formulae mentioned[15]. The plasma was obtained by centrifugation of blood at 3000 r/mn for 15 min and non-haemolysed plasma was stored in deep freezer for further biochemical analyses. Plasma glucose was determined using glucose kits supplied by Boehring Mannheium kit. Total serum protein content was determined calorimetrically. Activities of serum amylase and alkaline phospatase were determined calorimetrically. Lactate dehydrogenases and gama gultamic transaminase were measured by using diamond diagnostics kits. Serum glutamic oxalo acetic acid and serum glutamic pyruvic transaminase were determined calorimetrically[16,17]. Serum minerals Mg^{2+},Ca^{2+} and Cl^{-} were determined calorimetrically[18].

2.5. In vivo challenge test

After 60 d of feeding, the fish of each group were divided into two subgroups, the first subgroup of each treatment was challenged *i.p.* with pathogenic *A. hydrophila* (0.1 mL of 10^7 cells/mL) which was obtained from Department of Microbiology, Kamaraj College. The second subgroup was injected *i.p.* by 0.1 mL of saline as control. Both subgroups were kept under the observation for 7 d to record the survival rate daily.

2.6. Histopathology assessment

Samples of the gills and liver were collected for a patho-histological examination and fixed in 10% formalin. Afterwards, the samples were dehydrated in a series of ethanol solutions 50%, 70%, 80%, 90%, 95% and 100% for 1, 1, 2, 2, 1.5 and 16 h respectively. Each of the samples was then transferred into a xylene solution for another 30 min and then embedded in a solution of xylene wax for 1 h and 5-mm-thick section. Section was cut using a rotary microtome and quickly transferred onto a slide and kept in an oven at 40-50 °C for 24 h. Each sample was observed under a compound light microscope attached with a camera.

2.7. Statistical analysis

The data for haemotobiochemical and growth parameters were analyzed using one-way analysis of variance. And the difference in the means between groups were tested for significance at the 95% confidence level. P values < 0.05 were considered to be significant, using Duncan's multiple range test. All statistical analyses were performed using the SPSS software Package, Version 11.5.

3. Results

3.1. Growth performance

Table 2 shows that the T_3 weight gain (7.31±1.02) g, SGR (3.82±0.54)% and FCR (1.25±0.22)% of *M. montanus* fingerlings increased significantly when fed a diet containing 3% of probiotic. These values decreased significantly in fish group fed a diet of T_1 with weight gain (6.48±0.23) g, SGR (3.53 ±0.15)% and FCR (1.42±0.54)%, T_2 diet containing was found to be slightly better compared to T_3 with weight gain of (6.65 ±1.65) g, SGR (3.36±0.56)% and FCR (1.35±0.65)%. The growth performance was found to be poor in control with weight gain (4.91±0.45) g, SGR (2.32±0.07)% and FCR (0.96±0.17)% when compared to T_1 , T_2 and T_3.

Table 2

Effect of basal diet fed with different concentrations of probiotics.

Treatment	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%)	FCR (%)	Survival rate
Control	3.46±0.03^{a}	8.37±0.21^{a}	4.91±0.45^{a}	2.32±0.07^{c}	0.96±0.17^{a}	87%
T_1	3.45±0.05^{a}	9.93±1.32^{b}	6.48±0.23^{b}	3.53±0.15^{a}	1.42±0.54^{b}	89%
T_2	3.47±0.01^{c}	10.12±1.65^{a}	6.65±0.11^{a}	3.56±0.56^{a}	1.35±0.65^{a}	98%
T_3	3.45±0.04^{b}	10.76±2.17^{a}	7.31±1.02^{a}	3.82±0.54^{c}	1.25±0.22^{a}	100%

Mean±SD for triplicate feeding groups. Values in the same row with different superscripts are significantly different (P<0.05).

3.2. Haemotobiochemical changes

The results for the monitored haematological parameters of *M. montanus* fingerlings fed with probiotic diet after 7 d of infection with the pathogenic bacteria are presented in Table 3. Significant difference (P<0.05) were observed in the haematology parameters between the control and treatment with probiotic diet. Overall the haematocrit, haemoglobin B, red blood count, cholesterol, total serum protein, calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^{-}) and total Ig values followed a similar pattern. The values were significantly higher in the infected fish maintained on the probiotic diet. Conversely the erythrocyte sedimentation rate, white blood count and serum glucose concentration were significantly higher in the infected fish fed with probiotic bacteria. The erythrocyte sedimentation rate value in the fish groups infected with *A. hydrophila* and maintained on the probiotic diet did not significantly differ from both the probiotic and non-probiotic controls. The haemoglobin B and haematocrit values of the probiotic treatments infected similar to those of the non-probiotic control. The fish infected with pathogen and fed the probiotic diet on the other hand showed haemoglobin B and haematocrit values similar to those of

the control. The red blood count values of the fish infected with *A. hydrophila* and fed the probiotic diet did not differ significantly from the control whereas all the fish infected with pathogen and maintained on the non-probiotic diet showed significant differences in red blood count between probiotic and controls respectively. The serum total Ig concentration was significantly higher ($P<0.05$) in the infected fish fed the probiotic diet than in the counterpart groups maintained on the control. The serum glucose level was significantly higher in control diet than in the groups fed the probiotic diet. Significant differences ($P<0.05$) were observed in the immunological parameters between the control and treatment with probiotic diet. The overall serum amylase, alkaline phosphates, serum glutamic-oxaloacetic transaminase, serum glutamic pyruvic transaminase, lactate dehydrogenases and gamma-glutamyl transpeptidase were significantly higher on the probiotic diet (Table 4).

Table 3

Haematological parameters of *M. montanus* 7 d post infection and fed with non-probiotic and probiotic diets.

Parameters	Control	T_1	T_2	T_3
ESR (mm)	1.0 ± 0.2^{a}	1.2 ± 0.2^{a}	2.0 ± 0.3^{b}	1.0 ± 0.2^{a}
Haematocrit (%)	26.9 ± 1.5^{c}	26.0 ± 1.7^{c}	26.1 ± 0.6^{c}	29.0 ± 0.7^{d}
Haemoglobin (g/dL)	8.5 ± 0.5^{c}	8.1 ± 1.1^{bc}	8.0 ± 0.5^{bc}	9.0 ± 0.8^{cd}
RBC (cells×10^6 mm^{-3})	2.8 ± 0.1^{ef}	2.3 ± 0.4^{d}	2.1 ± 0.3^{cd}	2.4 ± 0.4^{de}
MCHC (g/dL)	31.7 ± 1.2^{a}	31.2 ± 1.7^{b}	30.6 ± 2.7^{b}	31.2 ± 2.6^{b}
MCH (pg/cell)	30.4 ± 1.7^{a}	33.8 ± 0.8^{b}	38.1 ± 0.9^{c}	37.7 ± 1.5^{c}
MCV (mm^{-3})	96.0 ± 3.0^{a}	113.0 ± 1.7^{b}	124.4 ± 1.0^{c}	120.9 ± 3.0^{c}
WBC (cell 5×10^3 mm^{-3})	24.3 ± 1.5^{a}	29.3 ± 0.6^{bc}	31.0 ± 1.0^{cd}	28.0 ± 1.0^{b}
Ig (mg/mL)	8.0 ± 0.6^{a}	9.7 ± 0.5^{b}	9.6 ± 0.1^{b}	9.8 ± 0.4^{b}
Total serum				
Protein (g/dL)	3.6 ± 0.2^{e}	3.3 ± 0.1^{d}	3.1 ± 0.1^{c}	3.4 ± 0.1^{d}
Serum glucose (mg/dL)	86.3 ± 2.4^{b}	129.9 ± 4.7^{c}	140.7 ± 6.7^{d}	124.7 ± 6.5^{c}
Cholesterol (mg/dL)	202.8 ± 8.0^{e}	178.0 ± 9.1^{cd}	167.2 ± 4.9^{c}	179.3 ± 3.5^{a}
Mg^{2+} (mg/dL)	3.4 ± 0.2^{d}	2.7 ± 0.1^{c}	2.6 ± 0.1^{c}	2.8 ± 0.1^{c}
Ca^{2+} (mg/dL)	12.1 ± 0.3^{c}	13.0 ± 0.2^{c}	12.9 ± 0.2^{c}	13.0 ± 0.2^{o}
Cl^{-} (mmol/dL)	134.3 ± 4.2^{d}	118.7 ± 6.5^{c}	112.4 ± 3.8^{bc}	120.3 ± 8.6^{c}

Mean±SD for triplicate feeding groups. Values in the same row with different superscripts are significantly different ($P<0.05$).

ESR: Erythrocyte sedimentation rate, RBC: Red blood count, MCHC: Mean corpuscular hemoglobin concentration, MCH: Mean corpuscular hemoglobin, MCV: Mean corpuscular volume, WBC: White blood count.

Table 4

Immunological parameters of *M. montanus* 7 d of post infection and fed with non-probiotic and non probiotic diets.

Parameters	Control	T_1	T_2	T_3
Serum amyl	7.24 ± 0.88^{a}	8.08 ± 0.93^{a}	9.65 ± 0.91	15.31 ± 1.20^{a}
Serum alkaline	0.39 ± 0.17^{a}	1.15 ± 0.02^{ab}	2.13 ± 0.13^{c}	3.15 ± 0.15^{a}
Phosphatase				
SGOT	51.26 ± 0.14^{c}	62.31 ± 0.24^{a}	65.36 ± 0.91^{a}	75.16 ± 1.02^{a}
SGPT	15.21 ± 0.99^{a}	27.2 ± 0.81^{ab}	29.3 ± 0.41^{a}	35.12 ± 0.54^{c}
LDH	176.5 ± 0.17^{a}	125.1 ± 0.49^{c}	115.3 ± 0.12^{a}	108 ± 0.58^{a}

Mean±SD for triplicate feeding groups. Values in the same row with different superscripts are significantly different ($P<0.05$).

SGOT: Serum glutamic-oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, LDH: Lactate dehydrogenases.

3.3. Histopathalogical changes

The histopathologic effects in the internal organs (liver and gills) of *M. montanus* are showed in Figures 1 and 2. When the specimens were collected at 7 d post infection with the pathogen compared with the very mild effects and observed in the infected fish fed the probiotic diet and control during the same period respectively.

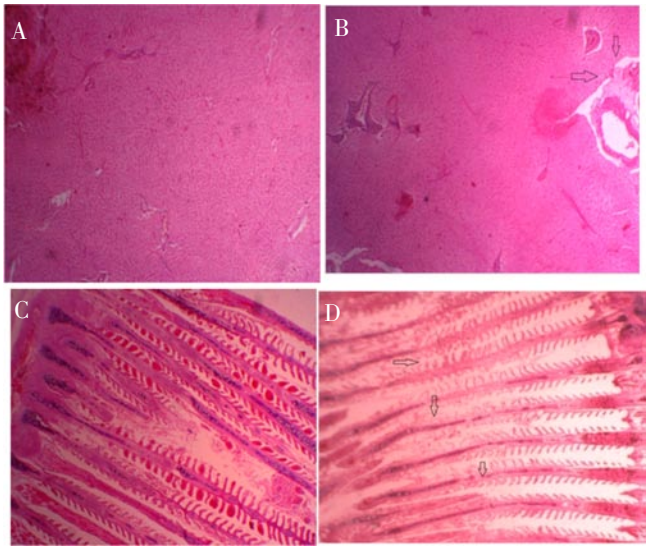

Figure 1. The histopathologic effects in the internal organs (liver and gills) of *M. montanus*.

A: Control liver (un infected); B: Liver infected with probiotic diet; C: Control gills (un infected); D: Gills infected with probiotic diet.

3.4. In vivo challenged test

Table 5 shows that the survival rate of T_3. A total of 99% of *M. montanus* fed on a diet containing 3% of *Lactobacillus* with challenged *i.p.* by pathogenic *A. hydrophila* (0.3 mL 0f 10^7 cells/mL). The survival rate of T_2 96%, T_1 94 % which fed on a diet containing 2% and 1% of *Lactobacillus*, this ratio was more than control 97%.

Table 5

Mortality rate of *M. montanus* fed diet containing probiotic bacteria for 60 d and challenged with *A. hydrophila*.

Items	Control	T1	T2	T_3
Route of injection	*i.p.*	*i.p.*	*i.p.*	*i.p.*
Dose of bacteria	0.1 mL of saline solution	0.1 mL (10^7 cells/mL)	0.1 mL (10^7 cells/mL)	0.1 mL (10^7 cells/mL)
No. of injected fish	10	10	10	10
Mortality	2	5.66 ± 0.57^{a}	3.66 ± 0.52^{b}	3.33 ± 5.72^{a}

4. Discussion

Probiotics which are micro-organisms or their products with health benefit to the host have been used in aquaculture as a means of diseases control supplementing or even in some cases replacing the use of antimicrobial compounds[19]. The present study showed that fish fed probiotic diet

showed significantly increase in SGR, FCR, weight gain and final weight of fishes compared to the control group. Similarly, *Carassius auratus* fed with probiotic diet were significantly restored the altered growth, haematological and immunological parameters, and triggered the innate immune system against *A. hydrophila*[20,21]. The final weight, weight gain, specific growth rate, survival rate feed intake and protein efficiency ratio were increased among *Oreochromis niloticus* fed with diet containing *Micrococcus luteus*[22]. The probiotic enhances growth rate of shrimps and maintains water quality parameters. Results of our study also correlated with studys in which shrimps were significantly greater in treated group compared with the control[23,24]. The use of probiotic can improve the nutrition level of aquaculture animal and improve immunity of cultured animals against pathogenic microorganisms. In addition, the use of Biogen® can reduce the frequency of outbreaks of disease[25]. The hematology observations in general could be due to infected fish fed with probiotic diet had a better capacity and lower stressor levels to resist infection compared with the other infected fish fed the non-probiotic diets. The higher erythrocyte sedimentation rate level noted in the present study could be related to damage to red blood count, due to the poison released by pathogenic bacteria during infection. The result higher than these values and were better in the probiotic treatments infected with control, which was possibly due to lower swelling and hence reduced red blood count damage in the probiotic treated groups[26]. Moreover, serum protein, cholesterol, Mg^{2+}, Ca^{2+} and Cl^{-} were significantly increased in infected fish fed with probiotics diets compared to control. The white blood count in the infected fish fed the non-probiotic diet was significantly higher than that in the infected fish maintained on the probiotic diet, which was possibly due to the induction of the non-specific defense and increased phagocytosis and cytotoxic activity in the infected fish maintained on the probiotic diet system[27]. Serum glucose was the highest in the group of fish fed the non-probiotic diet than the other groups as reported when tilapia was infected with *A. hydrophila*. The increased serum glucose level in the probiotic treatments observed in the present study could be due to fish requiring more energy to support all metabolic processes under abnormal conditions. However, in emergencies such as the stimulation of the immune defense system during bacterial infection, the fish could have also converted several lipids into glucose and this may explain the decreased cholesterol lever observed in this study. The observation in the present study showed that total Ig was significantly higher in infected fish fed the probiotic diet, which agrees with reports for trout, maintained on a probiotic based diet containing *Lactobacillus*[28].

In conclusion, the results of the present study clearly demonstrate that the infected groups of fish maintained on probiotic diets yielded significantly better growth, haematology parameters and histopathology than the control.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was performed in partial fulfilment of the requirements of a Master of Science in Zoology thesis of the first author, Kamaraj College, Manonmaniam Sundaranr Unviersity, Tamilnadu, India.

Comments

Background

The present study evaluated the protective effect of probiotic diets on haemotobiochemical and histopathology changes of *M. montanus* against *A. hydrophila*. The blood samples were collected for haematobiochemical analysis, while samples of the liver, and gills were examined for path histology after 7 d of infection. The result showed that the growth parameters, weight gain, specific growth rate were better in infected group maintained on the probioctic diet.

Research frontiers

In the present study, the *Lactobacillus* diet as a growth promoter and disease resistance against *A. hydrophila* were evaluated.

Related reports

Several related reported have been studied. Moeinfaramazi (2007) showed a significant effect of *Lactobacillus acidophilus* on better growth and immune function compared to the unchallenged diet.

Innovations and breakthroughs

Data from this study shows that challenged fish treated with probiotic diet yielded better growth and immune protection than the non-probiotic diet.

Applications

Lactobacillus acidophilus is the most commonly used to inhibit harmful bacterial against fish and humans. It helps in breaking down of food particles and helps to produce enzymes. It is mainly used for treating bacterial infection and growth production. The present study suggested that the probiotic diet helped to protect desease in cat fish against *A. hydrophila*.

Peer review

This is a well written study about the disease challenge of fish treated with probiotic diet. The results are clear and it is concluded that infected groups of fish maintained on probiotic diets yielded significantly better growth, haematology parameters and histopathology than the control. This article is very useful for fish diet against *A. hydrophila*.

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