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Research Article

TRIGGERING NON-SPECIFIC IMMUNITY OF *HETEROPNEUSTES FOSSILIS* AGAINST *AEROMONAS HYDROPHILA* USING HERBAL IMMUNIZATION

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ABSTRACT

Heteropneustes fossilis is considered as a cheap and manageable fish species for aquaculture. *Aeromonas hydrophila* was found as one of principal pathogenic bacteria causing diseases among the stinging catfishes. In the present study, herbal extracts like *Coleus aromaticus* (CA), *Mentha piperita* (MP), *Leucas aspera* (LA) and their combo (Tri Herbal Extract - THE) were utilized in enhancing the immunity of catfishes by intraperitoneal injection along with FKC vaccines against *A. hydrophila*. FKC vaccines with THE and CA were found to trigger the non-specific immunity, which was analyzed using hematological, biochemical and immunological studies.

KEYWORDS: *Heteropneustes fossilis*, FKC vaccine, *Aeromonas hydrophila*

INTRODUCTION

The most emerging food developing sector is aquaculture, in contrast with the earthly animal production, in which Asia's contribution is 90%[1]. Fish have been regarded extensively as a reticent fountainhead of protein enriched diet for human consumption from time immemorial. Moreover, fish farming adds to the economy of the nation by employments and enhancing the output of worldwide exchange[2]. Singhi is a standout amongst the most exceptionally requested freshwater air breathing fish species in the Indian subcontinent and Southeast Asian locale[3]. *H. fossilis* is considered as a manageable fish species for aquaculture. High demand and shopper inclination for singhi is because of less intra muscular spines, delicate flesh with delightful taste, high protein, iron and low fat substance and costs Rs. 300/Kg[4].

A. hydrophila is related with varied diseases of freshwater fishes. It is a vital pathogen in bringing about anxiety related infections in fish with the regular side effects of ulceration, exophthalmia and abdominal and known to be the cause of haemorrhagic septicaemia[5], Motile *Aeromonas* Septicaemia (MAS)[6] or Epizootic Ulcerative Syndrome (EUS) as an essential bacterial pathogen[7]. *A. hydrophila* were found to bring about diseases in fishes related with the fungus, *A. invadans* to create EUS[8]. *A. hydrophila* from EUS influenced singhi *H. fossilis* was recognized[9]. Histopathological changes in liver and kidney of the fish were influenced by this bacterium[10]. Experimental pathogenesis of *A. hydrophila* in singhi was studied[11]. Histopatholgical changes in experimentally singhi was examined with same bacteria[12].

In spite of the fact that being in part successful, the use of antibiotic agents and chemotherapeutants to control ailments has been broadly censured for its negative effects. Issues including solubility, agreeability, toxicity, cost, conveyance and legislative limitations have restricted the accessible antibiotics are as often as possible used in aquaculture to increment larval resistance; however the substances contaminate the ecosystem and aid in microbial resistance[13], residual accumulation in tissue[14] and fish immune suppression[15] bringing about the inconvenience of stringent controls to restrict the utilization of anti-biotic[16].

Immunization with inactivated antigens and in addition to probiotic or herbs in the feed of cultured fish may add to reduction in chemicals and antibiotics in aquaculture. Therefore, data on the pathogen and the host reaction must be considered for the advancement of a proficient immunization[17,18]. The resistance of fish can be assessed by breaking down the survival rate after experimental infection[19] and additionally hematological and immunological parameters[20].

In the present study, herbal extracts of *Coleus aromaticus, Mentha piperita, Leucas aspera* and their combo were studied for their immune boosting potential against *Aeromonas hydrophila* in stinging catfish, *Heteropneustes fossilis.*

MATERIALS AND METHODS

Isolation of pathogens from diseased fish samples

Adult fishes of *Heteropneustes fossilis* (196.97 ± 25.00 g and 18.53 ± 1.20 cm) with possible symptoms of EUS like unresponsiveness,

superficial lesions, swelling, and deep ulcer hemorrhages were collected from local fish markets of Tamiraparani river fed systems, Tirunelveli [8.44°N, 77.44°E] (Tamil Nadu, India) during north-east monsoon period and transported in aluminium drums to the Centre for Aquaculture Research and Extension (CARE) Aquafarm (St. Xavier's College, Palayamkottai) in live condition and acclimatized in cement tanks (12 m × 3 m × 2 m) for 24 h. After quantitative enumeration, the bacterial pathogens were subjected to standard microbial assays for further identification using KB002 HiAssorted Biochemical Test Kit (Himedia, India).

Toxic activities of Extra Cellular Products (ECPs) of *A. hydrophila* isolates *in vivo*

To prepare the ECPs of *A. hydrophila* isolates (Ah1, Ah14 & Ah21), the bacterium was grown in 20 ml TSB supplemented with catfish plasma at 10% and TSB w/o plasma served as control. All cultures were incubated at 30°C with 400 rpm. After 18 h, the broth cultures were centrifuged at 8000g for 10 minutes at 4°C to remove bacterial cells and the supernatants were filtered through 0.22-µm-pore membranes (Millipore) and stored at 4°C until use. Sterility was confirmed by streaking the filtered supernatants onto TSA plates[21]. Catfishes were injected with 200 µl of each ECPs (triplicates with six fish each group) and maintained for a week. Control fishes were injected with sterile TSB supplemented with plasma. The mortality was monitored for 7 days after injection and was recorded. Based on the obtained result, the most virulent strain was selected and used for further studies.

Determination of Lethal Dose ($LD_{50\text{-}96hr}$) of *A. hydrophila* isolate for disease challenge

Ah14 isolate of *A. hydrophila* was used for intramuscular injection of catfishes with 0; 1 x 10^5; 1 x 10^6; 1 x 10^7 and 1 x 10^8CFU/ml of *A. hydrophila* per fish[22]. Bacterial concentration that provoked 50% mortality in 96 h was utilized to challenge the fish.

Preparation of Formalin Killed Cell vaccines (FKC) with herbs

Overnight culture of *A. hydrophila* in Tryptic Soy Broth (TSB) was washed thrice with PBS and suspended in PBS with 0.4% formalin to a final concentration of 2.5×10^{10} cells ml^{-1}. Tri Herbal Extract (THE) was prepared in the ratio of 1:1:1 (*Coleus aromaticus, Mentha piperita, Leucas aspera*). The Formalin Killed Cells (FKC) thereafter diluted with an equal volume of saline (FKC vaccine) or with equal volume of Herbal extracts individually or in combination as THE (FKC-CA, FKC-MP, FKC-LA and FKC-THE vaccines respectively) at a concentration of 50 mg/ml. The vaccines were stored at 4°C until use.

Disease challenge experiment following immunization

H. fossilis (228± 19.8 g and 21.6 ± 1.9 cm) were acclimated in cement tanks for 10 days and fed ad libitum with a diet containing 28% crude protein twice a day. Immunization was done with slight methodology modification[23]. Before inoculation and for blood collection, fish were anesthetized with clove oil at 400 ppm concentration[24]. After vaccination on day 1, the fishes were challenged with 100 µl of 1 x 10^6 CFU/ml *A. hydrophila* in *H. fossilis* on day 7. All the experimental fishes were monitored for a month and subjected to various hematological, biochemical and immunological assays on day 15 and day 30. After the fish were anesthetized, the blood was withdrawn from the heart by cardiac puncturing method with 10% EDTA and a syringe without anticoagulant. Blood collected without EDTA was left to clot for 2 h at 25 ºC, centrifuged at 1400 g for 10 minutes and stored at -20ºC until analysis. Serum samples of three fish from the same experimental aquarium were pooled for immunological analyses[21].The mortalities were recorded and the Relative Level of Protection (RLP) among the challenged fish was determined[25].

RLP = 1 – [percentage of treated mortality / percentage of control mortality] × 100

Hematological, biochemical and immunological studies on challenged fishes

The following haematological parameters *viz*., Total Erythrocyte Count (TEC), Total Leukocyte Count (TLC) and Haemoglobin (Hb) were analyzed on initial (day 0), day 15 and 30. TEC (10^6 mm^{-3}) was determined by 1: 20 dilution ratio of the blood sample in Hayem's solution and TLC (10^4 mm^{-3}) with 1: 200 dilution ratio of the blood sample in Turkey's solution. The cells were counted in Neubauerhaemocytometer. Hb (g/dl) was determined by cyanhaemoglobin method[26]. Wright staining determined percent leukocyte count[27]. The percentage of neutrophil (NEU), eosinophil (EOS), lymphocyte (LYM) and monocyte (MON) were determined[28]. The derived blood indices of Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated[29]. Subsequently the biochemical indices including the Total Protein (TP; g/dl^{-1}), glucose (GLO; mg dl^{-1}) and cholesterol (CHO; $mmol/L^{-1}$) were determined[30].

NBT assay was used to measure the respiratory burst activity of the neutrophils using Secombes[31]. Plasma lysozyme and Phagocytic activity was determined[32,33]. Statistical analysis was performed using the one way analysis of variance (ANOVA). It was performed with SPSS statistical software (version 10.0, SPSS).

RESULTS

Isolation of pathogens from diseased fish samples

Fish samples which showed disease symptoms like necrosis, ulceration and itchy patches were examined for the quantitative and qualitative presence of bacteria in different organs and the results were noted as Total Heterotrophic Bacterial Count (THBC). The maximum load of bacteria was $8.9 \pm 0.7 \times 10^7$cfu g^{-1} in muscle and the minimum was $5.6 \pm 0.3 \times 10^4$ cfu g^{-1} in liver. Bacterial identification done using KB002 HiAssorted Biochemical Test Kit (Himedia, India) and the results obtained were tabulated (Table 1.1). The varied distribution of different bacterial species was articulated in percentage (Table 1.2). Among them, *A. hydrophila* was the principal bacteria secluded as the highest percentage as 59.1% with its maximum presence in muscle (38%) and minimum in intestine (3.7%). 21 isolates of *A. hydrophila* were identified from diseased fish samples and sorted our based on the presence of virulence genes (*unpublished data*). *In vivo* studies were carried out using ECPs retrieved from *A. hydrophila* isolates (Ah1, Ah14 & Ah21) to categorize the most virulent bacterial isolate for further investigations.

Toxic activities of Extra Cellular Products (ECPs) of *A. hydrophila* isolates *in vivo*

Ah1 ECPs resulted in the death of two out of 18 catfishes after 72 hrs. It was shown that the Ah14 extracted ECPs were toxic causing the loss of seven catfishes and the death percentage was recorded as 38.89% catfishes. In detail, four catfishes expired quickly within 5 hours of ECP injection, while the other fishes deceased later within 72 hrs. Though all the fishes were weak due to ECP injection, only a few catfishes recovered from their toxicity and exhibited normal movements.

While considering Ah21 ECPs, only one catfish died due to injection on 4th day, while the remaining fishes displayed sluggish behavior and were alive till the end of the experiment. All the control catfishes died and unveiled noble movement. Ah14 was declared as the most lethal isolate and thus utilized for further investigation.

Determination of Lethal Dose ($LD_{50\text{-}96hr}$) of *A. hydrophila* isolate for disease challenge

Considering lethal dosage study, the mortality transpired within 14 h after *A. hydrophila* challenge at a dosage of 1 x 10^7 CFU/ml in *H. fossilis*; while 1 x 10^8 CFU/ml caused mortality after 48 h of challenge. Some characteristic symptoms observed were patchiness, reddish swellings and also hemorrhagic descaling. 46.67%, 66.67% and 93.33% of mortality were observed cumulatively at varied dosages of 1 x 10^6, 1 x 10^7 and 1 x 10^8 CFU/ml among *H. fossilis* respectively. $LD_{50\text{-}96hr}$ of *A. hydrophila* in *H. fossilis* was calculated as 1 x 10^6 CFU/ml and used for challenging studies.

Disease challenge experiment following immunization

H. fossilis injected with 200µl of herbal vaccines on 1st day and challenged with *A. hydrophila* on 7th day. Hematological, biochemical and immunological assays of IU (Infected - Untreated), vaccinated groups *viz*., FKC, FKC-CA, FKC-MP, FKC-LA and FKC-THE *H. Fossilis* were carried out on the 15th day and 30th day of herbal vaccination. RLP of IU against vaccine immunized *H. fossilis*, revealed that the peak RLP was noted in FKC-THE treatments (65.22%) and FKC-CA treatment (56.53%).

Hematological, biochemical and immunological studies on challenged fishes

While studying the hematology, Infected-untreated *H. fossilis* showed a gradual decrease in HB, PCV, MCH, MCHC, RBC and NEU than that of the control fish. Considering the vaccine immunized and challenged *H. fossilis,* blood parameters *viz*., MCH, HB, MCHC, PCV, NEU and RBC level in FKC-MP, FKC-LA and FKC decreased significantly ($P<0.05$), whereas in FKC-CA and FKC-THE, the increased values were observed ($P>0.05$) and nearly control values on 30th day. For FKC-CA treated *H. fossilis*, RBC (Figure 2) (31.04+0.62 on 15th day to 28.17±0.1 on 30th day), Hb (Figure 1) (9.2±0.22 on 15th day to 9.5±0.33 on 30th day), MCH (44.6±0.37 on 15th day to 33.69±0.26 on 30th day), NEU (Figure 3) (45.5±0.5 on 15th day to 43.0 ±0.5 on 30th day), PCV (30.2±0.51 on 15th day to 36.4±0.32 on 30th day) and MCHC (25.09±0.21 on 15th day to 26.9±0.11 on 30th day) were noticed.

For FKC-THE treated *H. fossilis*, RBC (Figure 2) (31.76 ±0.21 on 15th day to 32.74±0.3 on 30th day), Hb (Figure 1) (9.56±0.10 on 15th day to 11.2±0.27 on 30th day), MCH (46.54 ± 0.31 on 15th day to 34.25±0.76 on 30th day), PCV (31.67±0.20 on 15th ay to 41.8±0.34 on 30th day), NEU (Figure 3) (45.0±0.25 on 15th day to 50.5±0.5 on 30th day) and MCHC (26.71±0.49 on 15th day to 29.21±0.19 on 30th day) were examined, where the increased values were notified reaching normal values on 30th day.

Significant increase in values of WBC, LYM, MCV, MON and EOS ($P < 0.05$) were determined while comparing with that of the control on 30th day for FKC-THE and FKC-CA treated *H. fossilis*. FKC-THE treated fish exhibited the following results: WBC (Figure 4) (39.4±0.24 on 15th day to 40.71±0.61 on 30th day), MON (Figure 5) (4.5±0.5 on 15th day to 8.0±0.5 on 30th day), LYM (Figure 6) (49.5± 0.25 on 15th day to 39.5±0.25 on 30th day), MCV (154.8±2.03 on 15th day to 143.31±0.93 on 30th day) and EOS (Figure 7) (1.0±0.25 on 15th day to 1.5±0.25 on 30th day).

FKC-CA treated *H. fossilis*, WBC (Figure 4) (41.12 ±0.41 on 15th day to 43.4±0.72 on 30th day), MCV (162.5±2.02 on 15th day to 179.4±0.21 on 30th day), MON (Figure 5) (4.0±0.5 on 15th day to 6.5±0.25 on 30th day),LYM (Figure 6) (49.5±0.5 on 15th day to 43.5±0.25 on 30th day) and EOS (Figure 7) (1.0±0.25 on 15th day to 3.5±0.25 on 30th day) were shown.

IU *H. fossilis* exhibited a significant decrease in cholesterol CHO (mg/dl) (117.02±1.35 on 15th day to 98.28±1.37 on 30th day), total protein TP (g/dl) (4.37 ±0.29 on 15th day to 3.98 ±0.32 on 30th day) and glucose GLU (mg/dl) (53.76 ± 0.85 on 15th day to 49.28±0.57 on 30th day), whereas for control, the following values *viz*., CHO (208.3 ± 2.23), TP (7.42 ± 0.21) and GLU (75.09 ± 1.06) (Table 3).

FKC-THE treated *H. fossilis* showed the following values *viz*., CHO (189.31±0.39 on 15th day to 203.6±0.39 on 30th day), TP (6.89 ±0.36 on 15th day to 7.18 ±0.61 on 30th day) and GLU (70.37±0.22 on 15th day to 72.31±0.11 on 30th day). In case of FKC-CA, TP (6.44 ± 0.31 on 15th day to 6.95 ±0.81 on 30th day), CHO (180.2±0.18 on 15th day to 187.5 ± 1.29 on 30th day) and GLU (68.32 ± 0.51 on 15th day to 66.37±0.17 on 30th day) were found. TP, CHO and GLU values decreased significantly ($P<0.05$) in case of FKC, FKC-LA and FKC-MP, while FKC-CA and FKC-THE values increased nearing control values on 30th day (Table 3).

Alanine amino transferase ALT (U/L) (79.26 ±0.17 on 15th day to 86.7 ± 0.33 on 30th day) (Figure 8), Aspartate-amino transferaseAST (U/L) (321.06 ± 2.06 on 15th day to 336.1 ±2.33 on 30th day) (Figure 9) and Alkaline phosphatase ALP (U/L) (190.7 ±0.22 on 15th day to 204.37 ± 1.33 on 30th day) (Figure 10) of IU tested *H. fossilis* significantly increased ($P < 0.05$) while comparing the control values *viz*., ALT (32.27 ± 0.15), AST (238.02 ± 1.04) and ALP (141.29 ± 0.91).

For FKC-THE, ALT (40.1 ±2.3 on 15th day to 37.7 ±0.24 on 30th day) (Figure 8), AST (267.3 ±1.77 on 15th day to 246.3 ±2.17 on 30th day) (Figure 9) and ALP (166.4 ±0.29 on 15th day to 149.5 ±0.19 on 30th day) (Figure 10) were notified. FKC-CA tested *H. fossilis*, ALT (43.7 ± 0.44 on 15th day to 39.1 ±0.7 on 30th day), AST (254.2 ± 1.35 on 15th day to 248.6 ±1.33 on 30th day) and ALP (168.06 ± 2.56 on 15th day to 151.6 ±0.16 on 30th day) were found. ALT, ALP and AST values of FKC-LA, FKC and FKC-MP increased significantly ($P<0.05$) till 30th day, while FKC-CA and FKC-THE values increased and reached normal values finally.

Immunological parameters namely Phagocytic Activity PA (%), Lysozyme Activity LA (mg/ml) and Nitroblue Tetrazolium Assay NBT (nmoles O^{2-}) of Immunized and Disease Challenged *H. fossilis* were noticed. IU *H. fossilis,* PA (0.63±0.11 on 15th day to 0.83±0.18 on 30th day) (Figure 11), LA (2.1±0.42 on 15th day to 3.1±1.4 on 30th day) (Figure 12) and NBT (0.10±0.01 on 15th day to 0.8±0.03 on 30th day) (Figure 13) showed lower values than that of control, namely PA (1.42±0.22), LA (8.3±1.31) and NBT (0.12±0.04) respectively.

More values of PA, LA and NBT were determined for FKC-THE *H. fossilis;* LA (16.3±1.81 on 15th day to 19.2±0.9 on 30th day) (Figure 12) and PA (2.34±0.72 on 15th day to 2.51±0.9 on 30th day) (Figure 11) and NBT (0.45±0.01 on 15th day to 0.49±0.04 on 30th day) (Figure 13) than that of control. *H. fossilis* treated with FKC-CA also exhibited more NBT, LA and PA values but lower than FKC-THE values; PA (1.67±0.24 on 15th day to 2.18±0.7 on 30th day), LA (14.2±0.94 on 15th day to 12.6±1.1 on 30th day) and NBT (0.26±0.01 on 15th day to 0.42±0.02 on 30th day) than control.

PA, LA and NBT values showed slight raise ($P<0.05$) in FKC-MP, FKC-LA and FKC while values of FKC-CA and FKC-THE significantly increased ($P>0.05$) comparing the IU and control group.

DISCUSSION

Incidence of contagious diseases is the principal obstruction in the enhancement of intensive fish farming and finally influencing the output due to increased mortality and retarded growth. It has been described as the most serious feature distressing the culture of catfishes, shrimps, carps and other cultured and wild fishes resulting in 10-90% of total loss in production[34].

In the present investigation, diseased *H. fossilis* were primarily collected and examined for external wide blanching, severe redness, descaling, itchiness, necrosis and fin rots. They were subjected to quantitative and qualitative presence of bacteria in various organs of the diseased fishes. In *H. fossilis*, the minimum count of $5.6 \pm 0.3 \times 10^4$ cfu $g^{-1}$was noted in liver, whereas the maximum value of $8.9 \pm 0.7 \times 10^7$cfu g^{-1} was shown in muscle.

In accordance with our results, maximum microbial count was noticed in gills (8.7±1.1 x 10^6 cfug^{-1}) rather than in intestine (5.8±0.4x10^7 cfug^{-1}) of Tilapia (hydrid)[35]. Maximum microbial count was recorded in muscle ($6.3 \pm 0.4 \times 107$ cfu/ml) rather than gills ($5.7 \pm 0.6 \times 106$ cfu/ml)[36] . Microbial load was more in muscle rather than gills in diseased fishes of *H. fossilis, C. carpio* and *C. punctatus*[37]. Maximum microbial load in muscle (8.3 ±1.8 x 10^7cfug^{-1}) and minimum in gills (3.2 ± 1.6 x 10^6cfu g^{-1}) were observed for *C. striatus*[38].

In the present study, twelve isolates, *viz*., *E. aerogenes, A. veronii, P. fluorescens, A. hydrophila, S. boydii, V. parahaemolyticus, E. coli, P. mirabilis, V. alginolyticus* and *Y. pestis* were recorded in different organs of infected fishes of *H. fossilis*. While considering the bacterial load, *A. hydrophila* was the prime pathogen isolated (59.1%), with its highest presence (38%) in tissues of muscle and meagerly in intestine (3.7%). Their presence in descending order was that *A. hydrophila*, *P. fluorescens* (12.7%), *V. parahaemolyticus* (1.0%) and *Y. pestis* (1.0%).

Our results are in accordance with the observation of 25 microbial species in freshwater carp (Himachal Pradesh, India)[39] and 15 isolated bacteria from hybrid tilapia (Saudi Arabia)[35]. Muscle and gill tissue samples of *C. striatus* explored the presence of 17 microbial species[40]. Presence of one fungal and 19 bacterial species were identified from infected *H. fossilis*[36]. Similarly, 10 bacterial species detected from disease infected marine fishes, *Amphiprion sebae* and *A. ocellaris* were *V. cholera*, *E. coli*, *V. alginolyticus, Proteus* sp., *V. parahaemolyticus*, *Y. enterocolitica. A. hydrophila*, *Streptococcus* sp. and *P. aeruginosa*[41]. The occurrence of 13 bacterial species was explored from diseased *C. carpio* and *C. punctatus,* while only 12 bacterial pathogens were revealed from infected *H. fossilis*[37].

In spite of various chemical methods of treatment like antibiotics, no successful preventive measure is on hand till date[42] for septicaemic situations of *A. hydrophila*. As a result, great attention is being directed towards natural antimicrobial products as pathogen-control products. Minimizing the usage for antibiotics, scheming microbial food infectivity, enhancing shelf-life conservatory technologies to eradicate disagreeable pathogens and/or setback microbial spoilage, lessening the progress of antibiotic resistance pathogens or intensifying the immune cells of humans are in urgent need[43].

Substitute techniques, like phytotherapy, can be an additional device in fish illness management. In India, 500 therapeutic plant species are utilized to treat in people. Plants have been utilized as conventional medication since ancient time to control bacterial, viral, and fungal illnesses. As of late, studies have been started to assess the possibility of utilizing herbal medicines fish disease management administration[44]. Herbal medicines have negligible side effects, are effectively biodegradable, cheap and locally accessible, and extracts are effortlessly prepared. Hence, it is advantageous to utilize herbal extracts as an elective tool for controlling bacterial diseases in aquaculture[45].

Considering the ECPs of *A. hydrophila* isolates (Ah1, Ah14 & Ah21), 11.11%, 38.89 % and 5.56% were recorded for *H. fossilis*. Though all the fishes were weak due to ECP injection, only a few catfishes recovered from their toxicity displaying sluggish behavior and were alive till the end of the experiment. All the control catfishes died. Ah14 was declared as the most lethal isolate and thus utilized for further investigation.

Meanwhile, *in vivo* study on crayfish with ECPs of *A. hydrophila* B1 resulted in the death of four fishes out of six, two suddenly within 3 hr and 46 hr. Cytotoxic activity with remarkable cytoplasmic vacuolization was observed within 30 min of administration. Supernatant containing extracellular toxins caused death due to damage of the hemocytes or other cells, resulting in cellular immunityfailure[21].

LD_{50}- 96hr of *A. hydrophila* in *H. fossilis* was found as1 x 10^6 CFU/ml. Fish mortality rate was determined to be in direct proportion with concentrations of *A. hydrophila.* While looking into the literature, various investigators have determined varied values on diverse fish variety. $LD_{50\text{-}96hr}$value of *A. hydrophila* was calculated as 2.4 x 10^8cfu/ml against *C. striatus*[46]. LD_{50} value of *A. hydrophila* was determined as 9.6 x 10^7CFU/fish[11] and 2.4 x 10^6cfu/ml[47] in *H. fossilis*.

Table 1. Biochemical characteristics of bacterial isolates from diseased *H. fossilis*

S. No.	Biochemical tests	*Aeromonas hydrophila*	*Pseudomonas fluorescens*	*Vibrio parahaemolyticus*	*Enterobacter aerogenes*	*Klabsiella pneumoniae*	*Proteus mirabilis*	*Vibrio alginolyticus*	*Shigella boydii*	*Yersinia pestis*	*Aeromonas veronii*	*Salmonella enteritidis*	*Escherichia coli*
1	Gram's staining	–	–	–	–	–	–	– –	–	–	–	–	–
2	Motility	+	+	+	D	+	+	+	+	+	+	+	+
3	Citrate utilization	D	+	– –	+	+	D	–	–	– –	+	+	–
4	lysine utilization	D	–	+	+	+	– –	+	–	– –	+	+	+
5	ornithine utilization	–	–	+	+	– –	+	D	–	– –	+	+	D
6	urease activity	–	D	D	–	+	+	–	– -	–	– –	– –	–
7	phenylamine deamination	+	– –	– –	–	–	+	– –	–	– –	D	– –	– –
8	nitrate reduction	+	+	+	+	+	+	+	+	D	+	+	+
9	H_2S production	+	– –	– –	– –	– –	+	– –	– –	– –	– –	+	– –
10	glucose utilization	+	+	+	+	+	+	+	+	+	+	+	+
11	adonitol utilization	–	–	–	+	+	–	– –	– –	– –	– –	– –	– –
12	lactose utilization	–	–	–	+	+	–	– –	– –	– –	– –	– –	+
13	arabinose utilization	–	–	–	+	+	–	– –	D	+	– –	+	+
14	sorbitol uilization	– –	– –	–	+	+	–	– –	D	D	– –	+	+

Note: + Positive; - Negative; D 11 – 89% show positive result; Nd Not determined

Table 2. Microbial load (percentage) in various organs of diseased *H. fossilis*

S. No.	Genera	Muscle	Gill	Liver	Intestine	Total %
1	*Aeromonas hydrophila*	38	10.5	6.9	3.7	59.1
2	*Pseudomonas fluorescens*	4.3	3.5	2.8	2.1	12.7
3	*Vibrio parahaemolyticus*	0.8	0.2	-	-	1.0
4	*Enterobacter aerogenes*	0.6	0.4	-	0.9	1.9
5	*Proteus mirabilis*	0.3	0.1	-	1.1	1.4
6	*Vibrio alginolyticus*	0.7	1.5	0.1	-	2.3
7	*Shigellaboydii*	1.8	0.5	0.3	-	2.6
8	*Yersinia pestis*	0.6	0.3	-	0.1	1.0
9	*Aeromonas veronii*	2.6	0.3	-	0.2	3.1
10	*Escherchia coli*	1.6	0.8	0.5	-	2.9

Table 3. Changes in the biochemical parameters of different groups of *H. fossilis* following immunization and disease challenge against *Aeromonas hydrophila*(Values expressed as Mean ± S.D of 3 replicates)

S. No.	Biochemical tests	C	Day 15						Day 30					
			IU	FKC	FKC-CA	FKC-MP	FKC-LA	FKC-THE	IU	FKC	FKC-CA	FKC-MP	FKC-LA	FKC-THE
1.	Glucose (mg/dl)	75.09 ± 1.06	53.76 ± 0.85	55.48± 0.3 8	68.32 ± 0.51	66.41 ± 0.55	58.31 ± 0.77	70.37± 0.22	49.28± 0.57	56.19± 1.2	66.37± 0.17	60.73 ±0.33	63.19± 0.22	72.31± 0.11
2.	Cholesterol (mg/dl)	208.3 ± 2.23	122.4± 0.25	145.7 ± 1.93	180.2± 0.18	176.3 ± 0.31	158.1 ± 2.4	189.31 ±0.39	119.31 ±0.18	159.2± 0.48	187.5 ± 1.29	183.7 ±0.17	172.5± 0.56	203.6± 0.39
3.	Total protein (g/dl)	7.42 ± 0.21	4.37 ±0.29	4.97 ± 0.41	6.44 ± 0.31	6.21 ± 0.21	5.94 ± 0.54	6.89 ±0.36	3.98 ±0.32	5.36 ±0.27	6.95 ±0.81	6.3 ±0.37	6.02 ±0.37	7.18 ±0.61
4.	Albumin (g/dl)	5.3 ± 0.44	2.91 ±0.11	3.67 ± 0.22	5.4 ± 0.16	4.76 ± 0.12	4.25 ± 0.21	4.73 ±0.23	2.84 ±0.18	3.38 ±0.16	5.2 ±0.27	4.2 ±0.17	3.92 ±0.25	5.7 ±0.28
5.	Globulin (g/dl)	2.12 ± 0.34	1.46 ±0.61	1.3 ± 0.17	1.04 ± 0.15	1.45 ± 0.07	1.69 ± 0.28	2.16 ±0.47	1.14 ±0.92	1.96 ±0.22	1.75 ±0.11	2.1 ±0.52	2.1 ±0.19	1.48 ±0.10

Note: Control - no extract, no vaccine; Day 1 - Injection of vaccine + extract; Day 7 - Challenge with *A. hydrophila*; Day 15 and Day 30 - Blood sampling, Control (C), Infected untreated (IU), Formalin Killed Cells (FKC), *C. aromaticus* (CA), *M. piperita* (MP), *L. aspera* (LA), Tri Herbal Extract (THE)

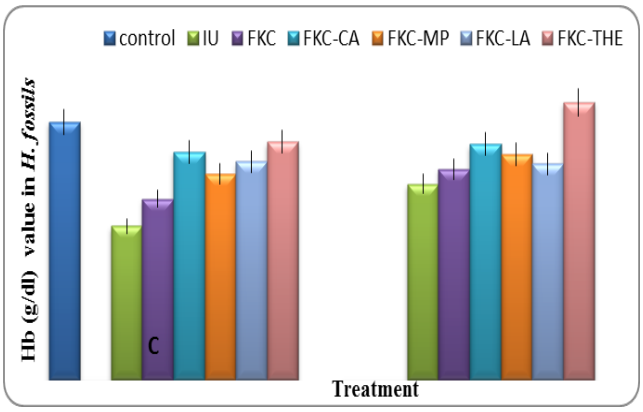

Figure 1. Haemoglobin values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

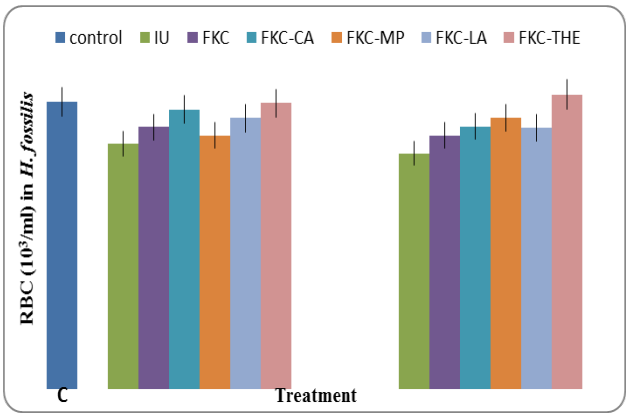

Figure 2. Changes in RBC values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

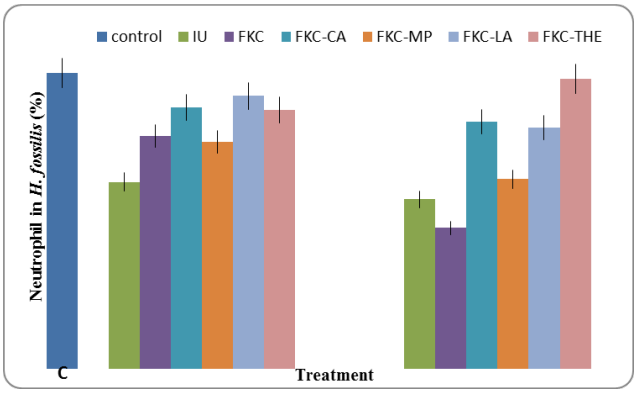

Figure 3. Neutrophil count of *H. fossilis* following immunization and disease challenge Against *A. hydrophila*

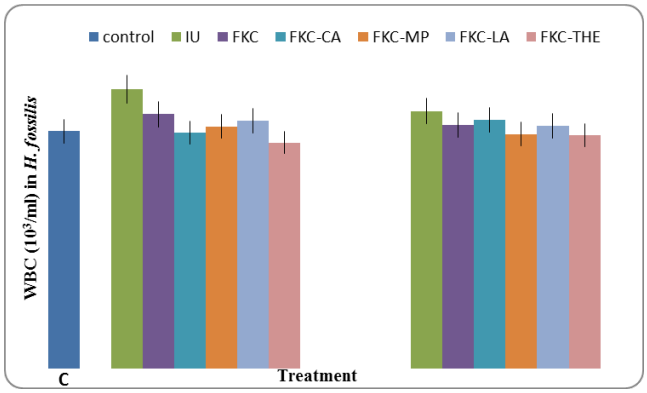

Figure 4. WBC values of *H. fossilis* following immunization and disease challenge Against *A. hydrophila*

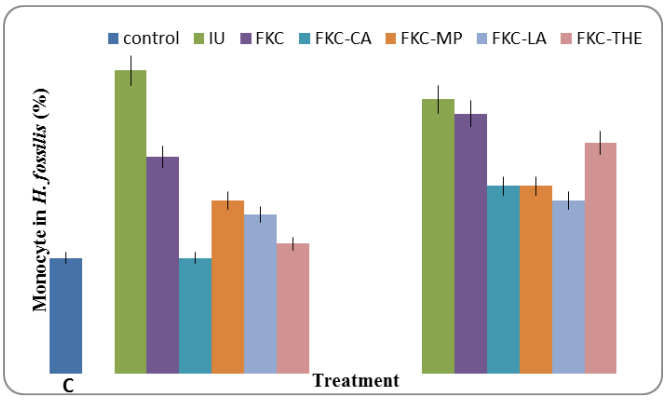

Figure 5. Monocyte count of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

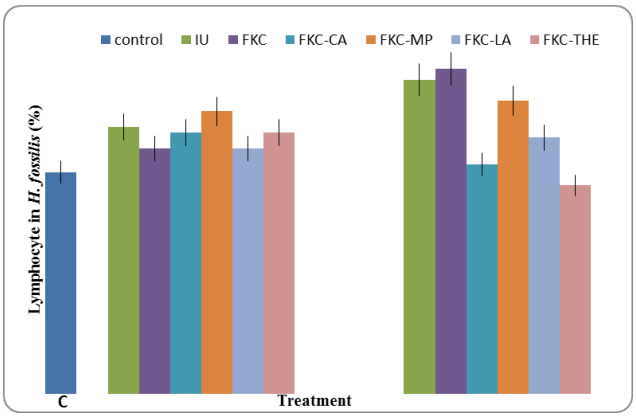

Figure 6. Lymphocyte count of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

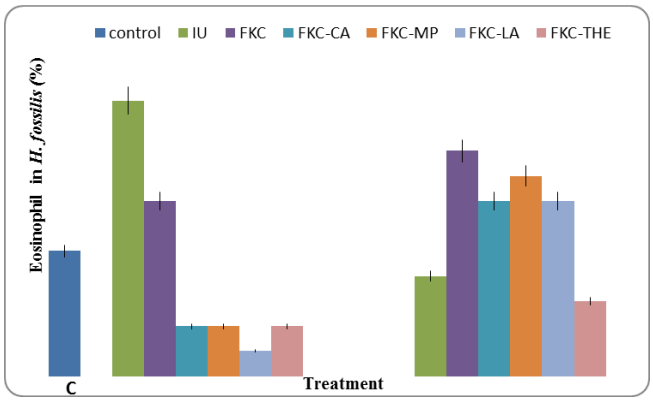

Figure 7. Eosinophil count of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

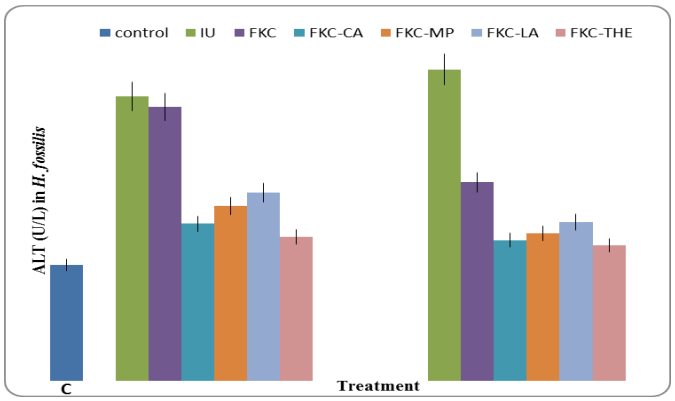

Figure 8. Changes in ALT values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

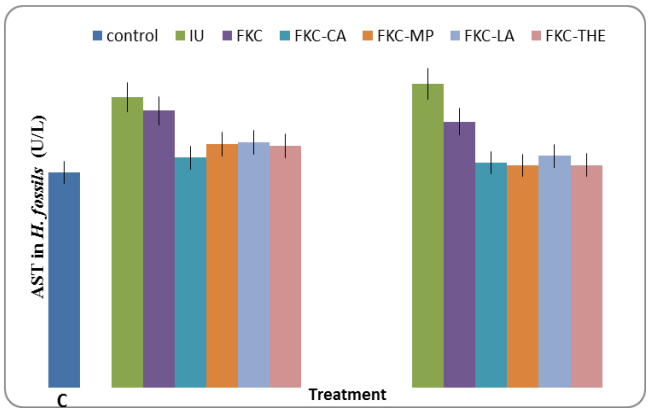

Figure 9. Changes in AST values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

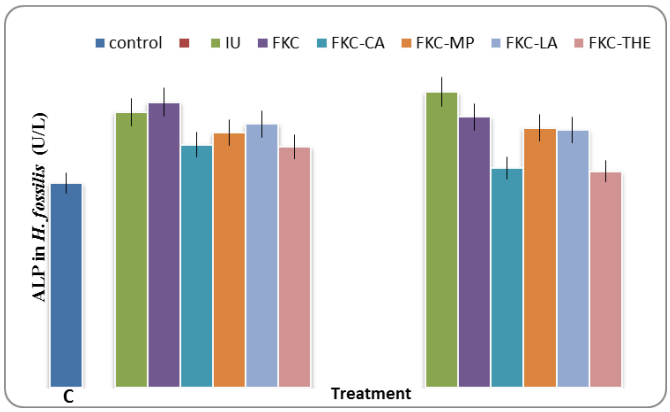

Figure 10. Changes in ALP values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

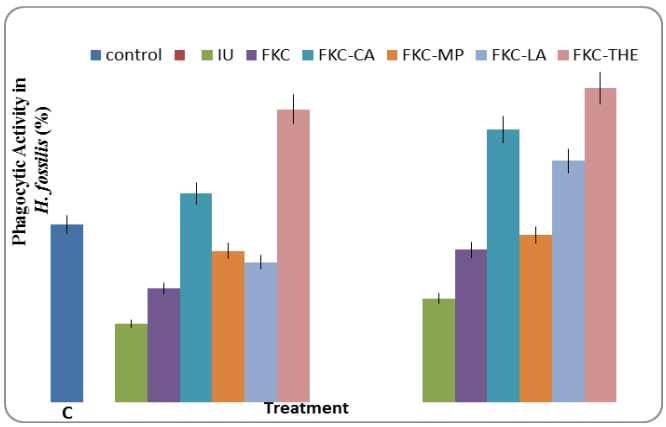

Figure 11. Changes in PA values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

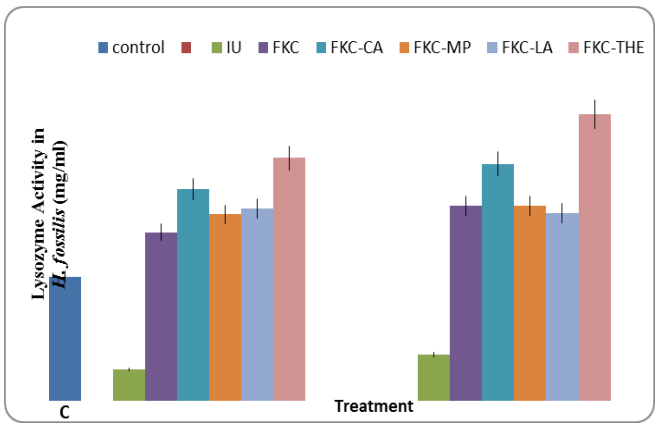

Figure 12.Changes in LA values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

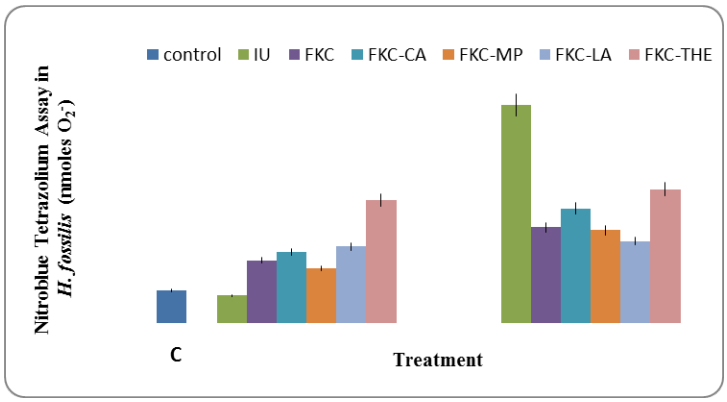

Figure 13. Changes in NBT values of *H. fossilis* following immunization and disease challenge against *A. hydrophila*

Highest level of RLP was determined as 65.22% in FKC-THE treatment followed by FKC-CA treatment as 56.53% in *H. fossilis* respectively. Considering disease challenge experiment, higher survival rates of 88.57% was determined in *C. carpio* fed with *O. basicilicum* diets comparing with control[48]. Similar trends were observed in *L. rohita*[49] and tilapia[50] fed with herb incorporated diets following challenge with *A. hydrophila.* Likewise, 50.59% RLP was determined in propolis incorporated diets than that of control against *A. hydrophila* in *Oreochromis niloticus*[25].

Hematological changes may imitate function of vital organs, equilibrium and homeostasis important for animal health valuation in aquaculture. Additionally, immunological assays in hand with molecular analyses also aid in accurate identification of ailments[51]. Varied humoral blood components portraying non-specific immune system were total serum protein (albumin and globulin), agglutinin, lysozyme and metalion-binding proteins are the major constituents of the non-specific immunity[51,52].

Immunostimulants can be widely used as adjuvants in vaccine preparation to initiate antigen presenting cells like macrophages and to activate more signal molecules like cytokines, which in turn kindle lymphocytes for production of specific immunoglobulins. Constituents like light oils, bacterial lipopolysaccharides (LPS) and Freund's adjuvants were depicted as generating increased antibody levels following injection along with bacterin[53].

Hematological, Biochemical and Immunological changes in *H. fossilis* were estimated following vaccine immunization and disease challenge with *A. hydrophila.*RLP of IU against vaccine immunized *H. fossilis*, revealed that the peak RLP was noted in FKC-THE treatments (65.22%) and FKC-CA treatment (56.53%). While studying the hematology, Infected-untreated *H. fossilis* showed a gradual decrease in HB, PCV, MCH, MCHC, RBC and NEU than that of the control fish. Considering the vaccine immunized and challenged *H. fossilis,* blood parameters *viz.*, MCH, HB, MCHC, PCV, NEU and RBC level in FKC-MP, FKC-LA and FKC decreased significantly ($P<0.05$), whereas in FKC-CA and FKC-THE, the increased values were observed ($P>0.05$) and nearly control values on 30th day.

Significant increase in values of WBC,LYM, MCV, MON and EOS ($P < 0.05$) were determined while comparing with that of the control on 30th day for FKC-THE and FKC-CA treated *H. fossilis*. TP, CHO and GLU values decreased significantly ($P<0.05$) in case of FKC, FKC-LA and FKC-MP, while FKC-CA and FKC-THE values increased nearing control values on 30th day. ALT, ALP and AST values of FKC-LA, FKC and FKC-MP increased significantly ($P<0.05$) till 30th day, while FKC-CA and FKC-THE values increased and reached normal values finally.PA, LA and NBT values showed slight raise ($P<0.05$) in FKC-MP, FKC-LA and FKC while values of FKC-CA and FKC-THE significantly increased ($P>0.05$) comparing the IU and control group.

Our results were in direct correlation with the investigations of various researchers. Herb incorporated diets were found as lessened the glucose levels, while increased Hb content, Hct level and WBC and RBC counts were identified in challenged fishes of common carp, *L. rohita*[54], *C. catla*[49] and *C. carpio*[55]. They also emphasized that the fish resistance increased using herbal extracts against the tested pathogens by enrichment and enhancement of the non-specific immunity of fishes. *Clarias batracus* showed increased response of both specific and non-specific immunity on diet incorporation of *O. gratissimum*[56]. They had showed a substantial decrease in the level of glucose while obvious rise was gotten in the levels of WBC, RBC, globulin and total protein representing a positive outcome for herbal supplementations.

Considering *C. auratus gibelio*, RPS in FKC + propolis group was expressively higher (67. 8%), than that of FKC (49.9%) against *A. hydrophila*[57]. Propolis was determined to have great synergism towards the development of vaccines. Antibody production, Phagocytosis activity, Leucocyte activity and survival rates were noted to exhibit striking increase in vaccinated fish related with unvaccinated and non-adjuvanated and groups following pathogenic challenge. The injection potential of methanolic extracts of *Ocimum sanctum*, *Solanum trilobatum* and their combo was explained[58]. The non-specific immunity i.e., increased levels of WBC count, phagocytic and serum antiprotease activity was observed with combo of both the herbal extracts. The adjuvant effects of *Aloe vera* in association with FKC and also Freund's adjuvant against *A. hydrophila*in common carp were studied in detail[59]. The immunological and hematological parameters *viz.*, WBC, NBT, serum lysozyme and alternative complement activities were displayed as highly significant ($P < 0.05$) in *Aloe* adjuvanated group rather than Freund's adjuvant.

CONCLUSION

Higher survival rate with very low mortality and increased levels of immunological parameters were recorded in adjuvant vaccinated groups of *H. fossilis* following pathogenic challenge. This strongly

projects the vaccine efficiency of natural adjuvants like herbs, to be used with greater protective effects for fish vaccination.

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