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Effects of dietary supplementation of *Pseudomonas aeruginosa* FARP72 on the immunomodulation and resistance to *Edwardsiella tarda* in *Pangasius pangasius*

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ABSTRACT

Edwardsiella tarda is one of the serious bacterial pathogens infecting both cultured and wild catfish urging an immediate need for effective protection strategies. This study assessed the effects of dietary supplementation of *Pseudomonas aeruginosa* FARP72 at 10^8 cells/g feed (PA diet) for 30 days on the innate immunity parameters, *viz.*, respiratory oxidative burst (ROB) activity, lysozyme, ceruloplasmin, myeloperoxidase, *in-vitro* nitric oxide (NO) production in addition to the expression of immune genes encoding interleukin-1β, C3 and transferrin in yellowtail catfish *Pangasius pangasius* and their resistance to *Edwardsiella tarda* challenge at a sub-lethal dose of 1.50×10^7 cells/fish. A significant increase in the innate immunity parameters was noted in PA diet-fed catfish on 30 dpf compared to the control. Post *E. tarda* challenge, the levels of immune parameters increased significantly and peaked at 5 dpi irrespective of feeding to confer protection against *E. tarda*. Their levels, however, decreased on and from 10 dpi. The results on the expression of immune genes encoding interleukin-1β, C3 and transferrin in the kidney and liver tissue samples of PA diet-fed *P. pangasius* upon challenge with *E. tarda* further confirmed the ability of *P. aeruginosa* to stimulate primary immune organs at the gene level. The effects of feeding *P. aeruginosa* FARP72 on the immune functions of catfish as examined by the functional immune assays, thus, demonstrating the innate immune responses of catfish that are differentially stimulated by the PA diet. The findings of our study would help evolve management strategies to confer protection against *E. tarda* infection in commercial catfish aquaculture.

1. Introduction

Globally, aquaculture plays a major role in the economy by its significant contribution to the income and wealth of people through the supply of nutritious food and by providing a fundamental source of livelihood, trade and recreation [1]. Indian aquaculture has shown a six and half fold growth in the last two decades, with freshwater aquaculture contributing over 95% of the total aquaculture production and more than half of the national fish production. Apart from freshwater aquaculture comprises of catfish, prawns and tilapia [2]. The farming of catfish took precedence over the last decade and pond farming became the dominant form due to its relatively faster growth rate, flesh quality and appearance thereby leading to its increased marketability overseas [3]. *Pangasius,* a fast-growing fish, has recently become a very popular food fish and a valuable aquaculture species in South-East Asia [4]. It has gained market share, particularly in the United States of America and to a lesser extent in the European Union [1]. An estimated amount of over 700,000 tonnes of catfish are produced in India per annum [2]. The

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farmed fish are more susceptible to diseases than their wild counterparts due to the artificial stressful conditions posed to them in intensive rearing practices [5]. Economic loss in catfish aquaculture due to diseases is considered to be the primary source of concern and bacterial infection continues to be a major threat for commercial catfish farmers [6]. Edwardsiella tarda is a serious bacterial pathogen infecting both cultured and wild fish. It has a broad host range and geographic distribution and contains important virulence factors that enhance host bacterial survival and pathogenesis [7]. In India, *E. tarda* infection has been reported in catfish *Clarias gariepinus* [8] and *Pangasius pangasius* [9] and carps *Catla catla* [10] and *Labeo rohita* [11,12].

With the changing scenario, diagnosis and prevention of infections are emphasized to promote health and production efficiency through chemotherapy, environmental manipulation, proper nutrition and immunological protection. However, the use of antibiotics and the emergence and spread of antimicrobial resistance have become a global threat to public health [13]. Presently, the most promising method of disease control in aquaculture is to strengthen the fish defense mechanisms. To date, much attention has been given to the immunomodulating effects of probiotics in aquaculture [14,15]. Yet, studies on the evaluation of immunomodulatory effects of the antagonistic bacterium with probiotic potential on the immune cells of tropical catfish are scarce. The isolation, identification and characterisation of *Pseudomonas aeruginosa* FARP72 with its antagonistic ability against fish pathogens [16] and protection against *Aeromonas hydrophila* infection in *Labeo rohita* [17] have been reported earlier. The present study aimed to investigate the effects of dietary supplementation of this bacterium on the immunomodulation in *P. pangasius* and protection against *E. tarda* infection.

2. Materials and methods

2.1. Bacterial strains

A bluish-green pigment-producing *Pseudomonas aeruginosa* FARP72 (NCBI Accession number KC570343) isolated from *C. batrachus* was used in this study. It proved its ability to fulfil the majority of the pre-requisite as a potent probiont [16]. It also exhibited *in-vitro* inhibitory activity against *Edwardsiella tarda* strains from diseased fish. A virulent catfish pathogen, *E. tarda* CGH9 (NCBI Accession number KX159725) was used for the experimental infection [9]. Before use, all strains were preserved at -20 °C in tryptic soy broth (TSB) with 15% sterile glycerol.

2.1.1. Ethical statement

The use of laboratory animals (fish) in the present study has complied with the guidelines and policies of the ethical committee of the Institute (Ref. No. FFS/Adm-6/2012/March 2012).

2.2. Pseudomonas aeruginosa FARP72 supplemented feed preparation

Pseudomonas aeruginosa FARP72 cell suspension was prepared as described in our earlier study [17] and the number of cells/mL of suspension on TSA was determined after incubation at 30 ± 2 °C for 48 h. Then, 5 mL crude cell suspension was mixed with 10 mL of vegetable oil and vortexed thoroughly. The bacteria-oil emulsion was then admixed with 1 kg pellet feed (CP 9910; Chareon Pokphand Private Ltd., India) to achieve a cell count of approximately 10^8 cells/g feed. The feed supplemented with *P. aeruginosa* cells (PA diet) was freshly prepared at a weekly interval. The control diet was prepared similarly by mixing 5 mL sterile saline with 10 mL of vegetable oil. The feeds were, then, uniformly spread, dried at room temperature (32±2°C) for 24 h and stored in airtight plastic containers.

2.3. Experimental catfish

Yellowtail catfish, *Pangasius pangasius* weighing 167.87±27.52 g were procured from a commercial fish breeder of Naihati (Lat. 22°54'10'' N and Long. 88°25'01'' E), North 24 Parganas district, West Bengal, India for assessing the immunomodulation in *P. Pangasius* upon dietary supplementation of *P. aeruginosa* FARP72. The fish, on receipt, were disinfected with 5 ppm potassium permanganate solution and acclimatized for 10 days at 40 fish/tank in circular fibreglass reinforced plastic (FRP) tanks of 500 L capacity containing 400 L bore-well water with continuous aeration. All fish were fed a commercial pellet diet at 3% of their body weight (BW) and maintained under optimal conditions.

2.4. Dietary supplementation of Pseudomonas aeruginosa FARP72

From the acclimatized stocks, *Pangasius pangasius* were distributed into groups viz., A and B. The fish of each group were stocked in FRP tank containing 300 L bore-well water at 20 numbers/tank in triplicate and fed with experimental diets at 3% BW in two divided doses for 30 days. The fish of group A were fed with a control diet and group B with a PA diet containing 10^8 cells/g feed. The fish of group A and group B were anaesthetized (0.1 mL clove oil/L water) on 30 days post-feeding and injected intramuscularly with 0.1 mL of diluted *E. tarda* CGH9 cell suspension adjacent to the dorsal fin to get a sub-lethal dose of 1.50×10^7 cfu/fish as described in an earlier study from our laboratory [9]. The sub-lethal dose taken was based on the lethal dose (LD_{50}: 3.87×10^7cfu/fish) determined during the previous experiment using *P. pangasius*. The research meets the ethical guidelines including adherence to the legal requirements of India.

2.5. Collection of fish blood and serum

The catfish were anaesthetized with clove oil (0.1 mL/L water) and blood was collected using a 2 mL sterile plastic syringe by caudal vein puncture from two fish of each replicate on every sampling. An aliquot of blood was heparinised using 2.7% EDTA and processed for measurement within an hour of collection. The non-heparinised blood was then allowed to clot at room temperature (≈30°C) by keeping the syringe in a slanting position and kept at 4°C overnight. The serum samples were collected by centrifugation at 2500 rpm for 15 min. The serum samples of two fish from each of the three replicates were pooled separately, labelled and stored at -20°C until use.

2.6. Head kidney (HK) leucocyte isolation and assessment of non-specific immune responses

After the collection of blood, the catfish were euthanized using clove oil (Dabur, India) at 0.4 mL/L water and the head kidneys were collected following the procedure described earlier [9,18]. Respiratory oxidative burst (ROB) activity by the neutrophils was determined by the reduction of nitroblue tetrazolium (NBT) to formazan [19]. The extent of NBT reduction was measured at an optical density of 540 nm using di-methyl formamide as blank. In-vitro nitric oxide (NO) production by macrophages was assessed based on the Griess reaction that quantified the nitrite content of the macrophage supernatants, as NO being an unstable molecule degrades into nitrite and nitrate [10]. The absorbance of the sample was measured at 595 nm in a microtitre plate reader (Dynamica, Australia). The NO production was quantified by its comparison to the sodium nitrite standard. The serum lysozyme content was assayed by a turbidometric method modified to a microtitre plate [20]. Following standard procedure, the initial optical density (OD) of the sample and the control was taken at 450 nm immediately after mixing the reagents. The final OD was taken after 1 h incubation at 30°C using a microtitre plate reader. Lyophilized hen egg-white lysozyme was used to develop a standard curve. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001/min and the result was expressed as serum lysozyme (units/mL).

The serum ceruloplasmin activity was determined as p-phenylenediamine (PPD) oxidase activity [19]. Following standard protocol,

the absorbance of the sample with its respective blank was measured at 540 nm using a microtitre plate reader. One unit of ceruloplasmin was defined as the amount of oxidase that catalysed PPD and caused a decreased spectral absorbance of 0.001/min at 550 nm. The serum myeloperoxidase activity was determined as per Sahoo and co-workers [19]. About 10 μL of serum was diluted with 90 μL of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then, 35 μL of freshly prepared 20 mM 3,3′,5,5′- tetramethylbenzidine hydrochloride [TMB] (Genei, India) and 5 mM H_2O_2 were added. The colour change reaction was stopped after 2 min by adding 35 μL of 4M sulphuric acid. The optical density was read at 450 nm in a microtitre plate reader.

2.7. Immune gene expression in Pangasius pangasius

2.7.1. Fish sampling for immune gene expression

Six fish from each group, i.e., two fish from each of the triplicate tanks were euthanized and sampled at identical time points on day 0, 30-day post-feeding (dpf), 1-day post-injection (dpi), 3 dpi and 10 dpi. The kidney and liver tissue samples were collected aseptically, placed in Trizol® reagent (Sigma, USA) and stored at -20 °C until further use.

2.7.2. RNA extraction and cDNA synthesis

The total RNA was extracted from different tissue (kidney and liver) using Trizol® reagent following the manufacturer's instructions. In brief, 0.1 g of each tissue sample was weighed and homogenized with 1 mL chilled Trizol®. RNA quality was checked in 2% agarose gel. Further, the concentration and the purity of RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The RNA was then treated with DNase I (RNase free; Thermo Fisher Scientific, USA) to remove the genomic DNA following the manufacturer's protocol. RNA (5 μg) was converted to cDNA using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) as per the protocol provided by the manufacturer. The cDNA was stored at -20°C for further use.

2.7.3. Primer design and nucleotide sequences

Amplification of C3, transferrin, interleukin 1-β (IL-1β) and β -actin (reference gene to check for the integrity of RNA) genes was performed by PCR using the earlier published primer sequences [21]. The sequences of the primers are given in Table 1.

2.7.4. Real-time quantitative PCR

qPCR was performed using the thermal cycler (Lightner 420, Roche, Germany). The 20 μl reactions contained 1 μL each of 5 μM forward and reverse primers, 10 μL SYBR Green qPCR Mastermix without ROX (Thermo Scientific, USA), 2 μL of cDNA template and 6 μL DEPC-treated H_2O. The reaction condition for qPCR was 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 57°C for 15 s, and extension at 72°C for 10 s. To confirm the specificity of amplification, melt curve analysis was performed at the end of the PCR reaction with a temperature ranging from 65 to 95 °C. The constitutively expressed reference gene, β-actin was used both as a positive control and for sample normalization. Each sample was performed in triplicate. The relative fold change was determined by the $2^{-\Delta\Delta CT}$ method [22].

Table 1
Primers used for the immune gene expression in *Pangasius pangasius*.

Gene name	Activity	Primer sequence(5'→3')	Product size (bp)
C3	Complement	F: TCCACCAGAGCCATCCCATA R: CACAACTTGAACGCCACCAG	198
Transferrin	Acute phase response	F: CACCCCATAACCTTCACCCC R: CGCAGTTTTCCCCAAACCAG	149
Interleukin-1β	Cytokine	F: CAGAGGCTGAAGCACACTCA R: CCTTGTCCTGCCTGCTGTAA	148
β-actin mRNA	Reference gene	F: ATTGATGCCCCTGGACACAG R: GGGTCTGTCCGTTCTTGGAG	133

Source: Sirimanapong et al. (2015); F: Forward; R: Reverse

2.8. Statistical analyses

Results of all experiments were expressed as mean ± standard deviation and analyzed using the Statistical Package for Social Sciences (IBM-SPSS) Version: 22.0, considering a probability level of $p < 0.05$ for the significance of the collected data. The results of the non-specific immune parameters of *P. pangasius* were analyzed by two-way ANOVA followed by an unpaired t-test for displaying statistical significance between the group at each time point. Also, the post-hoc homogeneity test in one-way ANOVA was done separately for each group to analyze the statistical significance among the group with time as a variable.

3. Results

3.1. Effect of dietary supplementation of Pseudomonas aeruginosa FARP72 on innate immunity of Pangasius pangasius

Dietary supplementation of *Pseudomonas aeruginosa* FARP72 significantly increased ($p < 0.05$) the innate immune response as reflected in the ROB activity, *in-vitro* NO production, ceruloplasmin, lysozyme and myeloperoxidase activities of *Pangasius pangasius* on 30 days post-feeding when compared to day 0 (Fig. 1a–e). All the immune parameters of both groups differed significantly on all days of post-challenge with *E. tarda* CGH9 ($p < 0.05$). The highest stimulation was recorded on 5-day post-infection (dpi) in PA diet-fed group ($p < 0.05$). From 10 dpi onwards, a significant decrease ($p < 0.05$) in the levels of immune parameters was observed in both groups. However, the extent of the decrease was more in control compared to the PA diet-fed group.

3.2. Expression of immune genes in Pangasius pangasius fed Pseudomonas aeruginosa diet

The results of the relative *in-vivo* gene expression of complement component C3, transferrin, and interleukin IL-1β transcript in the liver and kidney tissues are depicted in Fig. 2. The C3 and IL-1β transcripts were up-regulated slightly but significantly ($p < 0.05$) in the liver and kidney of PA diet-fed catfish on 30 dpf, whereas, the transferrin gene expression varied insignificantly ($p>0.05$) irrespective of diets. At 1-day post-infection with *E. tarda*, PA diet-fed catfish exhibited significant ($p < 0.05$) up-regulation of C3, IL-1β and transferrin transcript in both liver and kidney. The highest upregulation of C3 and IL-1β transcript was reported on 3 dpi and transferrin on 1 dpi compared to control. However, the magnitude of increase in C3, interleukin IL-1β and transferrin transcript was high in PA diet-fed catfish compared to control.

4. Discussion

The use of specific bacterium and biological compounds has been reported to stimulate non-specific host defense mechanisms, thus enhancing disease resistance and growth in hosts [23,49]. In this study, a significant increase in ROB activity by leucocytes of PA diet-fed *P. pangasius* demonstrated the ability of *P. aeruginosa* FARP72 in priming non-specific immunity in catfish. Macrophages and neutrophils produce bactericidal reactive oxygen species (ROS) during respiratory burst as a result of phagocytosis when stimulated by foreign substances [24]. A similar observation of stimulation in ROB activity after dietary

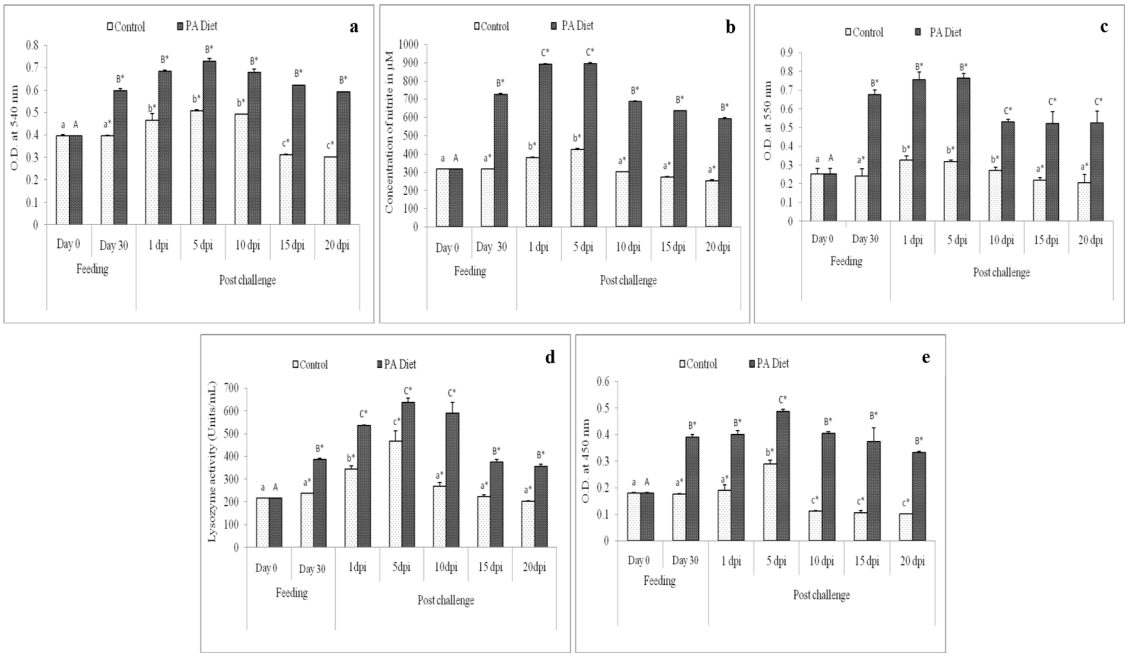

Fig. 1. Innate immune responses of *Pseudomonas aeruginosa* FARP72 (PA) diet-fed and *Edwardsiella tarda* challenged *Pangasius pangasius* as reflected by **(a)** respiratory oxidative burst activity, **(b)** *in-vitro* nitrite production, **(c)** serum ceruloplasmin, **(d)** lysozyme and **(e)** myeloperoxidase activities; Data are presented as mean ± standard deviation. a–c, A–C: Bars sharing uncommon superscripts within a particular group denote significant differences with time ($p < 0.05$). Bars sharing asterisks (*) at each time point differed significantly ($p < 0.05$). dpi: days post-infection.

probiotic supplementation in fish involving other feeding regimes and feeding durations was demonstrated earlier [25]. Typical clinical signs and symptoms described earlier in *E. tarda* challenged *P. pangasius* were noted. Moreover, no mortality was observed in the PA diet and control groups. Upon challenge with *E. tarda* CGH9, an increased ROB activity was observed in *P. pangasius* irrespective of feeding on 1–5 dpi, reaching the highest on 5 dpi in the PA diet-fed group possibly due to an increase in phagocytosis to attack the invasive pathogens. Increased killing capacity has been correlated with the enhanced ROB activity in trout [26]. The ROB activity reduced from 10 dpi onwards irrespective of feeding however, the reduction was least in PA diet-fed catfish. On 20 dpi, the ROB levels were significantly higher than on day 0, indicating extended protection and enhanced ability of the PA diet to eliminate *E. tarda*. These results also suggested the normalization of fish survival with time. The present study, however, contradicted the results of *E. tarda* challenged *L. rohita* [11], *C. catla* [10] and *P. pangasius* [9], which observed a decreased ROB activity immediately after infection, possibly due to the ability of *E. tarda* to survive within the host phagocytic cells by producing enzymes like superoxide dismutase and catalase to neutralize the effects of ROS [27].

NO production, which plays a major role in the host-defense response, significantly increased in *P. pangasius* fed PA diet on 30 dpf. The observed levels of NO produced were comparable to those recorded in *P. pangasius* [9]. Irrespective of feeding, the levels of NO increased from 1 dpi with *E. tarda* CGH9 and peaked at 5 dpi, probably due to the increased ability of catfish to fight against the infectious agents [28]. The cell wall components of *P. aeruginosa* FARP72 and their synergistic influence on the overall outcome for the cell function possibly triggered the innate immune signalling cascade, leading to the expression of inducible NO synthase (iNOS) and synthesis of NO. It has been reported that iNOS production serves as the first line of defense in Ictalurus punctatus [29]. The increase in NO production was possibly associated with more burden of oxidative stress and the ability of the fish to fight against the pathogen entry similar to those observed in *P. pangasius* on 5 dpi with *E. tarda* [9] and other catfish [30]. Nevertheless, the decrement was least in PA diet-fed catfish, thus, signifying the enhanced and prolonged immunomodulatory activity of *P. aeruginosa* FARP72.

The serum ceruloplasmin activity in the control and PA-diet-fed *P. pangasius* was lower than in an earlier observation [9], which may be due to individual variations reflecting the overall health status. It is known that the increase in ceruloplasmin activity may be associated with the general well-being of fish [31]. The serum ceruloplasmin activities increased significantly on 30 dpf in PA diet-fed *P. pangasius*. In *E. tarda* challenged catfish, a significant increase in ceruloplasmin was recorded from 1 dpi with a peak on 5 dpi, which then decreased on 10 dpi irrespective of feeding. In contrast, the serum ceruloplasmin levels of *E. tarda*-challenged catfish started decreasing from 15 dpi onwards compared to control [9]. Several other studies also demonstrated an increase in ceruloplasmin activity in fish infected with pathogenic bacteria [19,20,28] suggesting its potential involvement in eliminating the pathogens. The decrease in ceruloplasmin activity on 10 dpi was the least in PA diet-fed catfish. Similarly, an earlier study demonstrated that the expression of ceruloplasmin after *A. hydrophila* infection in *L. rohita* was significantly up-regulated during infection, which, however, decreased to the initial level on 15 dpi in the survivors [31]. They further documented the highest ceruloplasmin expression on 3 dpi. Our study, however, noted the highest ceruloplasmin activity on 5 dpi with *E. tarda* in PA diet-fed catfish. The ceruloplasmin activity reached the level observed at 30 dpf in control catfish at 15 dpi. In contrast, a higher ceruloplasmin level was observed in PA diet-fed catfish even on 20 dpi, which further confirms a better and prolonged immunomodulatory activity of the PA diet.

The serum lysozyme activity of the catfish on day 0 of this study was lower than the levels observed in *P. pangasius* [9] possibly due to the individual variations in fish. Upon injection with *E. tarda* CGH9, the lysozyme levels increased from 1 dpi onwards with a peak on 5 dpi and a significant decrease on and after 10 dpi. In contrast, a steady increase in the lysozyme activity of *E. tarda*-challenged catfish was reported up to 15 dpi [9]. Likewise, a significant increase in serum lysozyme levels was reported in *Silurus glanis* [32], *C. catla* [10] and *L. rohita* [11] up to 15 dpi with *E. tarda*. The increase in lysozyme indicated lysozyme secretion as the first line of defense by the granulocytes of *P. pangasius* during non-specific oxygen-independent responses against *E. tarda* infection. The significantly higher lysozyme levels as observed in PA diet-fed

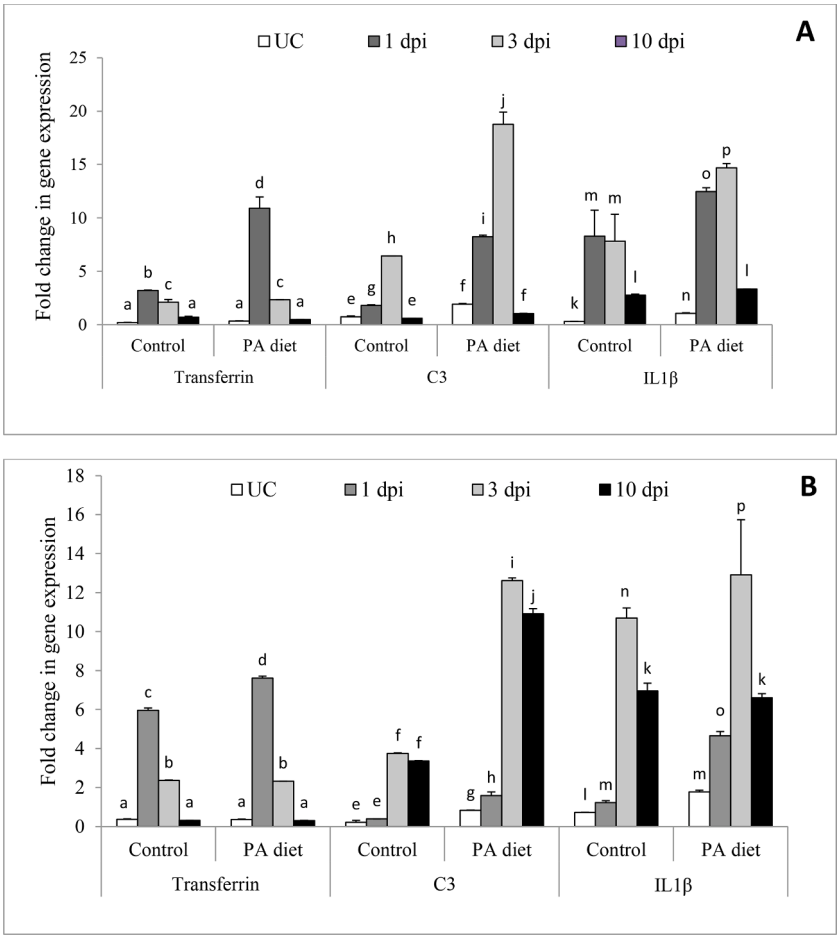

Fig. 2. Relative fold-expression of transferrin, complement C3 and interleukin 1β gene in kidney (A) and liver (B) of *Pseudomonas aeruginosa* FARP72 (PA) diet-fed and *Edwardsiella tarda* challenged *Pangasius pangasius*. Data are presented as mean ± SD. Different superscripts (a, b, c, d, e, f, g, h, i, j, k, l, m, n, o) above the bars in each series denote significant differences among treatments within the specific gene/organ ($P < 0.05$). dpf: days post-feeding; dpi: days post-infection

P. pangasius even on 20 dpi compared to control or day 0 suggested the immunopotentiating role of *P. aeruginosa* FARP72 in combating the *E. tarda* infection.

In PA diet-fed catfish, the magnitude of increment in MPO activity was highest, indicating the ability of the bacterium in increasing the innate immune activity of catfish similar to an earlier study [15]. In *E. tarda* challenged catfish, the MPO levels increased from 1 dpi onwards with the highest on 5 dpi irrespective of feeding and subsequently decreased on and after 10 dpi. Likewise, in an earlier study, the MPO activity in *E. tarda-challenged* catfish increased significantly at 5 dpi, reducing thereafter [9]. Alike ROB, lysozyme and ceruloplasmin activities and NO production, the MPO levels of PA diet-fed catfish were significantly higher on 20 dpi than on day 0. Collectively, it is clear from the present study that the dietary administration of *P. aeruginosa* FARP72 primed the non-specific immune parameters of *P. pangasius* better and offered prolonged resistance to *E. tarda* infection. These results, thus, demonstrated that the innate immune responses of *P. pangasius* are differentially stimulated by the PA diet.

The effects of different immunostimulants on the expression of immune genes are reported earlier in fish [33,34] including *Pangasianodon* hypophthalmus [35], but not in *P. pangasius*. The complement system plays a key role in the innate and adaptive immunity mediating phagocytosis, respiratory burst, chemotaxis and cell lysis [36]. The present study noted a significant up-regulation in the gene encoding complement component C3 in the liver and kidney of PA diet-fed *P. pangasius* compared to the control. The fold increase was almost 2.9 and 3.36 times higher in PA diet-fed catfish kidney and liver, respectively. The results implied a higher up-regulation of the C3 gene in the kidney compared to the liver of PA diet-fed and subsequently, *E. tarda* challenged catfish. Moreover, the results suggested a vital role of these organs in innate immunity and defences against *E. tarda* invasion in catfish. In contrast, the highest C3 gene expression was reported in the liver of dark barbel catfish Pelteobagrus vachellii than in the kidney and blood [37] and other fish [38]. Also, the C3 gene expression was noted only in the liver of olive flounder when stimulated with LPS [39]. The distribution difference of the C3 gene in the kidney and liver as observed in our study and an earlier study [37] may be due to the configuration of the varied isotypes. Perhaps, *P. pangasius* and other catfish may have more C3 isoforms, as in Atlantic cod and halibut [40], which warrants further studies on the C3 isoforms of *P. pangasius*.

Statistically, an insignificant difference was observed in the expression profile of the C3 gene in the kidney of *P. pangasius* at 30 dpf and 10 dpi irrespective of feeding, implying that the expression of the C3 gene reached the normal level at 10 dpi. Interestingly, the MPO activity also reached a normal level at 10 dpi. The MPO is capable of inducing properdin-initiated C3 *in-vitro* [41]. Likewise, a rise in C3 transcripts at 12 and 24 h post-challenge with *E. tarda* compared to control was documented [11]. Furthermore, an up-regulation in the expression of transferrin, intelectin, toll-like receptor 5, complement C3, ceruloplasmin and fibrinogen was demonstrated in the liver of *I. punctatus* infected with a Gram-negative bacterium at 3 dpi [42]. Contrarily, no significant difference was recorded in the expression levels of complement C3 and IL-1β genes in the liver of uninfected and *E. ictaluri*-infected groups of *P. hypopohthalmus* [35], though there was an increase in the

infected groups fed with immunostimulant diets. The results of the present study corroborated the observations on the up-regulation of the C3 complement component in *Oncorhynchus mykiss* [14, 34], *L. rohita* [43], *L bata* [44] and *Pimephales promelas* [45] fed with different immunostimulants or provoked by different pathogenic agents. Mohammadian and co-workers [46] reported the effects of autochthonous probiotics on the expression of some immune-related genes of *A. hydrophila* infected *Tor grypus*. Similarly, our results also depicted the role of *P. aeruginosa* in augmenting the immune responses of *P. pangasius* as an immunostimulant.

Interleukin-1β reportedly induces a cascade of effects leading to inflammation and release of cytokines and is responsible for an increase in phagocytosis, lymphocyte proliferation and superoxide anion production [47]. There is no clear understanding of the effects of probiotic treatment on the production of inflammatory cytokines. The present study noticed an increase in IL-1β gene expression in the liver and kidney of PA diet-fed catfish on 30 dpf. Likewise, enhancement in the expression of IL-1β was observed after 15 days of administration of probiotic *L. acidophilus* [48], however, the increment recorded was lower than in the present study. Several earlier studies also demonstrated significant enhancement of TGF-β and IL-1β with *Lactobacillus* and *Enterococcus* [49], expression ratios of IL-1β and TNF-α during in-vitro co-incubation of HK leukocytes of *O. mykiss* with *Carnobacterium maltaromaticum* B26 and *C. divergens* B33 [50] carp IL-1β and iNOS mRNA in the blood when injected with two secondary metabolites (4-trans-hydroxy-L-proline and cyclo-(L-Pro-Gly2)) of *Bacillus simplex* [51]. In an earlier study, the expression of IL-1β was significantly up-regulated by 3.89 folds in 6 h post-infection (hpi) with *E. tarda* that returned to normal levels at 7 dpi [11]. Similarly, in this study, significant up-regulation of IL-1β was observed in the liver and kidney of *P. pangasius* from 1 dpi with *E. tarda,* which then decreased to the initial level at 10 dpi. Interestingly, the magnitude of increase in the expression profile of IL-1β transcript was more in the kidney (12.47 folds) than in the liver (4.66 folds). Likewise, differential expression of cytokine in the pathogen aggravated *L. rohita* following immunostimulation with levan was reported [52].

The results of the present study evidenced that the highest and statistically significant up-regulation of IL-1β (14.68 folds) was in the kidney of PA diet-fed catfish on 3 dpi, while in the liver, the fold change was 12.91. Similarly, a higher level of expression of IL-1β was reported in the anterior kidney of immunostimulant diet-fed and *E. tarda* challenged *P. hypophthalmus* [35], probably due to a higher concentration of macrophages and neutrophils present in this tissue than in other organs. Also, significantly high expression of IL-1β in the head kidney and spleen compared to the gut or liver of *I. punctatus* during *E. ictaluri* infection [53], in spleen and head kidney of *Paralichthys olivaceus* up on immersion challenge with *Nocardia seriolae* suspensions [54] and in the liver, head kidney, spleen, intestine, and muscle of *I. punctatus* infected with *E. ictaluri* [55] have been well documented. The results of Ho and co-workers [56] also confirmed significantly higher expression (up to 9 folds) of IL-1β in the spleen and kidney at 3 dpi in Micropterus salmoides injected with a virulent strain of *N. seriolae*, which was down-regulated at 5 dpi. The observations on the up-regulation of IL-1β suggested that the *P. aeruginosa* FARP72 may involve in anti-infective and anti-stress functions during infection similar to the levan molecule [52]. The present study also noted a decrease in the expression of IL-1β transcript from 10 dpi, which corroborated the findings of an earlier study in β-glucan fed P. hypophthalmus [21]. They reported an insignificant up-regulation of the IL-1β gene from 15 dpi with *E. tarda* in the kidney of *P. hypophthalmus* fed β-glucan diet. In contrast, an earlier study documented about 7.5 folds increase of IL-1β in 2 hpi over control, which remained higher up to 8 hpi when the adult zebrafish were intraperitoneally injected with *E. tarda* [57]. Also, the macrophages from β-glucan and LPS treated *C. carpio* [33] and aloe vera fed *O. mykiss* [47] did not increase the transcript expression of IL-1β. Our results strongly suggested that the IL-1β plays an important role in catfish defence during *E. tarda* infection.

The regulation of iron homeostasis is an important aspect of the acute phase response in catfish [42]. Transferrins, primarily found in the liver, are physiologically important multitask globular proteins involved in iron-binding and transport, anti-microbial activity, growth differentiation and cytoprotection processes [58]. In this study, the transferrin gene expression varied insignificantly in the liver and kidney of PA diet-fed *P. pangasius* on 30 dpf compared to the control. Similarly, insignificant differences in transferrin gene expression were noted in the gill, spleen and kidney of turbot given a nucleotide-supplemented diet than control [59]. In contrast, a significant increase in transferrin gene expression profile in the spleen and kidney of *O. niloticus* fed *L. acidophilus* supplemented diet for 15 days was reported [48]. Furthermore, in *E. tarda* challenged catfish, a significant up-regulation in transferrin transcript was observed in the liver and kidney of catfish from 1 dpi onwards irrespective of feeding. The up-regulation was the highest (7.62 folds) in the liver of PA diet-fed catfish on 1 dpi. While in the kidney, 6.92 folds increase in transferrin transcript was noted. The expression of transferrin transcript decreased significantly from 3 dpi onwards in catfish irrespective of feeding. These results validated the earlier reports on the maximum expression of transferrin on 1 dpi with *A. hydrophila* in *O. niloticus* fed *L. acidophilus* [48] and *E. tarda* in *L. rohita* fed lipopolysaccharide [44]. Similar to this study, the highest transferrin gene expression was noted in the liver of I. punctatus compared to most other tissues [60]. The present study noted a reduction in the expression of the transferrin transcript gene from 3 dpi onwards. Nevertheless, the reduction was the least in PA diet-fed catfish. Contrarily, a significant up-regulation of transferrin transcript on 3 and 7 dpi with *E. ictaluri* was demonstrated [60]. They opined that transferrin act as a positive acute-phase protein in fish to increase iron storage to make it unavailable for bacterial growth. It, however, served as a negative acute phase response in *P. hypophthalmus* during the early stages of infection with *E. ictaluri* [61]. Nevertheless, the results on the immune gene expression along with the non-specific immune parameters of PA diet-fed *P. pangasius* upon challenge with *E. tarda* confirmed the ability of *P. aeruginosa* FARP72 to stimulate primary immune organs at the gene level and immune effector activities. The findings of the present study would help evolve management strategies to protect catfish against *E. tarda* infection in commercial aquaculture.

5. Conclusion

Augmenting India's fish production to 20 million tonnes by 2022–23 is the national priority. In this study, administration of *P. aeruginosa* FARP72 at 10^8 cells/g feed and feeding for 30 days improved the immune responses and overall health of catfish *P. pangasius*. This approach can be used to produce safe and disease-resistant catfish, which would help reduce antibiotic usage in aquaculture, thus, avoiding the development of antibiotic resistance. Yet, the performance of *P. aeruginosa* FARP72 under intensive culture needs to be studied to assess their beneficial effect in field conditions.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

All authors of this paper have read and approved the final version submitted. The contents of this manuscript have not been copyrighted or published previously.

(1) The contents of this manuscript are not now under consideration for publication elsewhere.

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(3) Use of laboratory animals (fish) in the present study has complied with the guidelines and policies of the ethical committee of the Institute (Ref. No. FFS/Adm-6/2012/March 2012).

CRediT authorship contribution statement

Farhana Hoque: Conceptualization, Visualization, Methodology, Data curation, Formal analysis, Validation, Funding acquisition, Writing – original draft. **T. Jawahar Abraham:** Conceptualization, Visualization, Supervision, Funding acquisition, Writing – review & editing. **S.N. Joardar:** Visualization, Validation. **Prasenjit Paria:** Formal analysis, Data curation. **Bijay Kumar Behera:** Visualization, Validation. **Basanta Kumar Das:** Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known conflict of interest that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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