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Starvation: An Alternate Measure to Improve Immunity and Physiology of Red Sea Bream During *Edwardsiella Tarda* Infection

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Abstract

Dietary restrictions during infectious challenges are quite common in animal kingdom. In the present investigation, we aimed to explore the positive implications of short-term starvation in *Edwardsiella tarda* infected red sea breams. Starvation resulted in depleted transcription of several iron binding protein *(Hepcidin, Transferrin)*, which could have reduced the bacterial colonization in starved- infected fish. This was confirmed by the significantly ($P<0.05$) low bacterial load in the spleen and muscle of starved-infected fish. Gills showed mild damage to the secondary filaments architecture as well as elevated mucus production in the starved-infected fish compared to the fed ones. Massive mucus cell hyperplasia was observed in starved-placebo fish, which further increased after infection. Decreased activities of serum anti-oxidative enzymes and reduced total antioxidant capacity after starvation was suggestive of improved stress response and heightened stress withstanding capacity of these fish. Relatively higher haemoglobin and phagocytic activity along with the increased cytokines *(TNFα, IL- 1β)* level in starved-infected groups than their fed counterparts indicated the better immune condition of the former group. Additionally, our data also demonstrated that starvation enhanced the survivability and overall disease resistance index of infected fish, indicating that short period of starvation might be a beneficial measure to fight against infections.

Keywords: Starvation; *Edwardsiella tarda*; Red sea bream; Cytokines; Gill mucus production; Iron homeostasis

Introduction

Edwardsiella tarda, a gram negative pathogenic bacteria, is known to affect both fresh and marine aquaculture of many economically valued fish, especially tilapia (*Oreochromis niloticus*), carp *(Labeo rohita)*, channel catfish *(Ictalurus punctatus)*, red sea bream *(Pagrus major)* and turbot *(Psetta maxima)* [1-4]. Despite the success of vaccination in fish disease prevention, till date, no effective vaccine has been developed and commercially marketed for *E. tarda* control [4]. In addition, plenty of reports suggest that the effect of *E. tarda* varies between fish species [5]. Hence in such complicated situation, the exigencies for alternative preventive measures increase exponentially in order toantagonize the losses incurred due to *E. tarda* infection.

The voluntary curtailment in food intake during infection is known to increase the survivability in various organisms [6], as excessive nutrition is often detrimental for immune homeostasis [7]. Anson et al. [8] reported that the infected mice that were unable to reduce their food intake suffered increased mortality, while the intermittent fasted mice showed increased glucose metabolism, neuronal resistance and lesser susceptibility to injury. Although the details vary across host species and type of pathogen [9], non-acceptance of food during any infection is highly modulated by the host's own immune-neural connections [10], and allows the animal to manipulate its complex physiological interconnections into a state that supports enhanced immune function [11].

Food restriction or starvation, and infection are both instrumental in changing various metabolic pathways in Atlantic salmon [12]. Reduction of food intake triggers the general defense response of fish, allotting energy or other resources to defeat the illness. Upon fasting, significant reduction in the rate of mortality was observed in the *Vibrio salmonicida* infected Atlantic salmon *(Salmo salar)* [13] and *Edwardsiella ictaluri* infected Channel catfish [14]. Food restriction is also known to reduce the free metal ion circulation in the body due to subdued intake. Since free iron is very essential for the growth and propagation of infectious agents, its curtailment might be an essential tool in reducing the rate of infection and further increasing the survivability of the fish. Starvation also results in theinduction of *Hepcidin* (an antimicrobial peptide and iron regulator) level, which further enhances the iron sequestering mechanism, leading to a reduction in the free iron content in the body. The dual function of *Hepcidin* as an antimicrobial peptide and iron homeostasis regulator establishes the linkage between the infection, iron metabolism and nutrient availability. *Hepcidin* induction was also found be strongly induced by the pro-inflammatory cytokines in mice, after viral and bacterial infections [15]. Numerous studies show that change in macrophages, cytokines and their associated signaling after calorie restriction is helpful in decreasing the chronic inflammation [16].

Red sea bream is a highly valued fish and an excellent candidate for commercial aquaculture as well as sports fisheries. However, in recent years, red sea bream aquaculture has been plagued by numerous diseases, with *E. tarda* infection being the most prevalent [5]. Thus, in the present investigation, we have tried to exemplify the effect of starvation on the immune profile, transcriptional alterations, gill tissue integrity and survivability of *E. tarda* infected red sea bream.

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Materials and Methods

Laboratory culture and preparation of pathogen

Pure strain of *E. tarda* was obtained from Ainan Town Office (Fisheries Research Division), re-grown on Salmonella-Shigella (SS) Agar media (Nissui Pharmaceutical, Japan) and used for inoculum preparation using previously published protocol [17]. Concisely, the SS agar media was dissolved in distilled water and centrifuged at 200xg for 1 min in order to remove the agar. The supernatant (SS liquid broth) was collected and autoclaved. Freshly grown pure bacterium was inoculated into the SS liquid media, incubated for 72-96 h at 28°C, and thereafter centrifuged at 2400xg for 15 min at 4°C to harvest the *E. tarda*. The pellets were thoroughly washed in phosphate buffer saline (PBS; pH 7.2), re-suspended in PBS, and the optical density at OD600 was measured and also subsequently diluted to quantify the number of viable colony forming units (CFU) by spread plate method [18]. Ultimately, a bacterial concentration of 10^9 CFU mL^{-1} was added to the water.

Fish husbandry and experimental design

Four month old red sea breams (24g ± 0.24) were procured from Yasutaka Suisan Company, and conditioned in flow-through sea water system with commercial diet (Otohime EP_2; Marubeni Nisshin Feed Co. Ltd., Japan). Two weeks after conditioning, the fish were transferred to eight different tanks (Tank 1-8) with two replicates each (stocking density of 20 fish per tank). The experimental fish were kept in 500L capacity flow-through fiber-reinforced tanks (300 L water holding capacity) with a flow rate of 1L min^{-1}. The water temperature in all the tanks was maintained at 25-26°C throughout the experimental period. Half of the fish (Tank 1-4) were fed twice a day, at the rate of 2.5% of body weight for 10 days, while the other half (Tank 5-8) were continuously starved. After 10 days, half of the fed (Tank 3 and 4) and starved group (Tank 7 and 8) fish were immersed in *E. tarda* infected water (final concentration 10^9 CFU mL^{-1}) for 5 h and named as fed-infected and starved-infected, respectively. The remaining fish were immersed in seawater containing sterilized SS media and renamed accordingly (Tank 1 and 2, fed-placebo; Tank 5 and 6, starved-placebo). Following pathogenic/placebo infection, the fish were grown in pathogen-free flow-through seawater for 10 more days, following the same feeding regime under 12h light and 12h darkness cycle. All the groups were immediately sampled after 5 h of immersion (0 dai (days after infection)) and further timely sampled on the 5th and 10th days after infection (represented as 5 and 10 dai, respectively).

Sample collection

At each sampling point, four fish from each group were anesthetized with clove oil (50 µL L^{-1}) (Wako, Japan) and blood was collected using a heparin-coated syringe. The blood was kept in heparin- coated eppendorf tubes and immediately used for analysis. For serum, blood was collected without any anti-coagulant, and transferred to non-heparin-coated eppendorf tubes. The blood was allowed to clot for 3 h and thereafter centrifuged at 3500xg for 15 min. The supernatant was collected and stored at - 30°C for future use. Immediately after blood collection, the unconscious fish were euthanized by cervical transection followed by pithing method, and dissected to collect the organs for various analyses. Portions of liver, spleen and kidney were stored in RNAlater for Real-time PCR analysis, and some were used for enzymatic analysis. Parts of gills were kept in Bouin solution for the histological observations.

Bacterial population analysis

DNA was isolated from the samples (stored at -80°C by snap-freezing) using DN easy Blood and Tissue kit (Qiagen, USA), following manufacturer's protocol. After quantification of DNA, 10ng of each sample was analyzed by Real-time PCR using SSO fast probe mix and *E. tarda* specific primers (Supplementary Table 1), following manufacturer's protocol.

Blood and serum analysis

The circulating blood glucose and total haemoglobin contents were assessed using Glucose colorimetric assay kit (Cayman, USA) and haemoglobin colorimetric assay kit (Cayman, USA), respectively, using manufacturer's instructions. The Phagocytic activity (the respiratory burst activity, NBT) was measured using fresh blood samples following previously published protocol (Mohapatra et al., 2014a).

Total protein, Albumin, SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase) and Total anti-oxidation capacity (TAC) were, respectively, measured using Protein assay kit (Abnova), BCP albumin assay kit (Abnova), SOD assay kit-WST (Dojindo, Japan), Catalase assay kit (Abnova), GPX assay kit (Biovision, USA) and Total antioxidant capacity assay (TAC) kit (Biovision, USA) kits, following the manufacturer's protocol. The globulin amount was calculated by deducting the albumin amount from the total protein content (19). The small molecule anti-oxidation (SMC) and protein anti-oxidation capacity (PAC) were further calculated from the TAC values.

Parameters		Placebo			Mean Placebo	Infected			Mean Infected	Overall Mean	Significance (P<0.05) by three way ANOVA						
		T0	T5	T10		T0	T5	T10			St	T	I	StT	StI	TI	StTI
CAT	Fed	0.01 ± 0.00^{ab}	0.00 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{A}	0.01 ± 0.00^{ab}	0.02 ± 0.00^{b}	0.03 ± 0.00^{c}	0.02 ± 0.00^{B}	0.01 ± 0.00^{X}	S	S	S	NS	NS	S	S
	Starved	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{A}	0.01 ± 0.00^{a}	0.02 ± 0.00^{ab}	0.02 ± 0.00^{ab}	0.02 ± 0.00^{B}	0.01 ± 0.03^{Y}							
	Mean	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00^{M}	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00^{N}								
SOD	Fed	0.03 ± 0.00^{b}	0.05 ± 0.01^{bc}	0.03 ± 0.00^{ab}	0.04 ± 0.00^{B}	0.07 ± 0.00^{c}	0.05 ± 0.00^{bc}	0.07 ± 0.01^{c}	0.06 ± 0.00^{D}	0.05 ± 0.00^{X}	NS	S	S	S	NS	S	NS
	Starved	0.02 ± 0.00^{a}	0.03 ± 0.00^{ab}	0.04 ± 0.00^{b}	0.03 ± 0.00^{A}	0.00 ± 0.00^{ab}	0.08 ± 0.02^{c}	0.07 ± 0.00^{c}	0.05 ± 0.01^{BC}	0.04 ± 0.00^{X}							
	Mean	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00^{M}	0.03 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.01^{N}								

Data expressed as mean ± SE (n = 4). All significance are measured at P<0.05. Different small superscript (a, b, c, d, etc.) represent the level of significance among different treatment groups at different sampling points for a particular parameter. Different capital superscript (A, B, C) represents significant difference between means of different experimental groups (fed-placebo, starved-placebo, fed-infected and starved-infected groups). Overall mean value having different capital superscript (M & N, X & Y) vary significantly. S-Significant, NS-Non-significant. Units: Catalase (CAT) – units mg $protein^{-1}$; Superoxide dismutase (SOD) – units mg $protein^{-1}$

Table 1: Effect of starvation (St), *E. tarda* infection (I), treatment time (T) and their interactions on gill stress enzymes activity in *P. major*.

Transcriptional profiling

Interleukin 8 (*IL8*, HM453864.1), Interleukin-1β (*IL-1β*, AY257219.1), Major Histocompatibilty Complex IIA (*MHCIIA*, AY698064.1), Tumor Necrosis Factor α (*TNFα*, AY314010.1), *Hepcidin 1* (AY452732.1), *Hepcidin 2* (AY669383.1), *Transferrin* (AY335444.1), Natural resistance-associated macrophage protein (*NRAMP*, AY485311.1) and ribosomal protein L8 (internal control, AY190734.1) sequences were obtained from National Center for Biotechnology Information (NCBI). All these genes were initially PCR amplified from liver cDNA (prepared by superscript III, Invitrogen, USA) using gene specific primer (Supplementary Table 1) and cloned into pGEMTeasy vector. One positive clone of each gene was used to prepare the Real-time PCR standard.

RNAs were isolated from the samples stored in RNAlater using Quazol (Quigen, USA), following manufacturer's protocol. gDNA eliminator solution was used to remove the genomic DNA contaminations. After quantification of RNA, 1μg RNA of each sample was used to prepare the cDNA using superscript III vaio kit (Invitrogen, USA) and then diluted to a final volume of 100μL. 1μL of prepared cDNA was used for Real-time PCR analysis of each gene. The Real-time PCR conditions included initial denaturation at 94°C (2 min) followed by 40 cycles of 94°C (30 s) and 60°C (1 min). Relative expression was calculated using L8 expression (internal control). PCR was performed on a Geneamp PCR system 9700 thermal cycler (Applied Biosystem, USA), using gene specific primers (Supplementary Table 1).

Histological analysis

The gills of the sampled fish were dissected free from the head and fixed in Bouin solution for 24 h at 4°C and then preserved in 70% ethanol and stored at 4°C until further use [19]. Later the samples were dehydrated (using a series of 70%, 90% and 100% ethanol), cleared in lemosol and embedded in paraffin. These were sectioned (4 μm) using a Leica RM2255 microtome (Leica Microsystems, Germany), mounted onto SuperFrost MAS-coated glass slides (Matsunami, Japan) and dried at 37°C for 24 h. The sections were stained with Mallory-Heidenhain's Azan staining method [20] with slight modifications. Briefly, sections were deparaffinized with xylene, rehydrated using graded ethanol, re-fixed in mordant solution, and serially stained with Azocarmine G. Excess stain was removed by Aniline-ethanol and Acetic-ethanol wash. Further, the slides were re-incubated with 5% Phosphotungstic acid hydrate solution, stained with Aniline blue-Orange G solution, dehydrated with ethanol (100%) and mounted in mixed solution of Canada balsam and xylene.

Two-colored immunohistochemistry (IHC)

The fixed and paraffin embedded gill samples were sectioned (10 μm thickness), deparaffinized,rehydrated and processed using previously published protocols [21]. The tissue fragments were then blocked in 2% blocking solution (Roche, Germany), incubated overnight at 4°C with Annexin V-Alexa 488 (Invitrogen, USA) and Beclin1-Alexa 561 (Abgent, USA) antibodies (both 1:500 dilution). Annexin V and Beclin1 antibodies, respectively, are known to depict strong signals in the apoptotic and autophagic cells of red sea bream. The antibody-coupled gill sections were then rinsed with PBST (PBS with 0.01% Tween 20), and analyzed or photographed using Confocal microscope (LSM 710, Zeiss, Germany).

Survivability

The fish health was monitored on a daily basis, using mobility, swimming behavior, body color, scale erosions, morbidity and food intake as principle wellbeing status criteria. Mortality was observed for all the groups for the entire experimental period. The mortality aspects were approved by the Animal Ethics and Use Committee of Ehime University, Japan. Sampling of the surviving fish was humanely (mentioned above) carried out at 0, 5 and 10 dai. Survivability was calculated using the following formula:

$$\%\text{Survivability} = \left[\begin{array}{l}\text{Total number of remaining fish at previous}\\ \text{sampling point} - \text{total number of fish died}\\ \text{between two sampling points}\end{array}\right] \text{X } 100 / \text{ Total number of remaining fish at previous sampling point}$$

Note that for 0 dai, the initial stocked number of fish was used for calculating the % survivability.

Disease resistance index (DRI)

An overall DRI was calculated using the following formula:

$$DRI = \frac{1}{n}\sum_{i=1}^{n}\frac{|X_i - Xref_i|}{Max\{|X_i - Xref_i|\}} * 100$$

Where Xi is the value of the i^{th} variable.

Xrefi is the reference value of the variable i which indicates its measurement at a healthy state.

The reference value indicating a healthy state differs from one variable to another according to the direction of variation depending on our preliminary analysis and available literatures, i.e. lower value indicates healthy state for some variables, such as the bacterial count, and higher value for other variables, such as the survivability rate. Thus, taking into consideration the two directions forcalculating the DRI, the Xrefi was considered as the maximum values, Max{xi} for the first group of variables and the minimum value Min{xi} for the second group.

Thus, each variable that entered the DRI was normalized to be between 0 and 100 and the DRI is interpreted as follow: The more the index, the better the overall resistance disease and vice versa.

Statistical analysis

One-way or Three-way analysis of variance (ANOVA) and Tukey's test was used for data analysis and significance calculation between the treatment means, respectively, using SPSS (Statistical Package for Social Sciences, Version 22.0) software. All experimental data are depicted as the mean ± SE. Differences were considered statistically significant at $P<0.05$, if not otherwise mentioned.

Results

E. tarda population and iron regulatory genes expression

The highest amount of relative *E. tarda* content was noticed at 0 dai in spleen and muscle of both the starved-infected and fed-infected groups (Figure 1). Although gradual decrease in the bacterial population was observed in both the tissues of the fed-infected group at 5 dai onwards, nearly no bacterial count was observed in the starved-infected groups at both 5 and 10 dai.

In spleen, the genes regulating the iron homeostasis, viz., *Hepcidin 1, Hepcidin 2, Transferrin* and *NRAMP*, showed a similar starvation related alteration (Figure 2). At 0 dai, the starved group showed a significant ($P<0.05$) increase in the expression of all the four studied genes, which however reduced drastically from 5 dai onwards. No significant ($P>0.05$) difference in expression pattern was observed between the infected and non-infected groups in the studied genes.

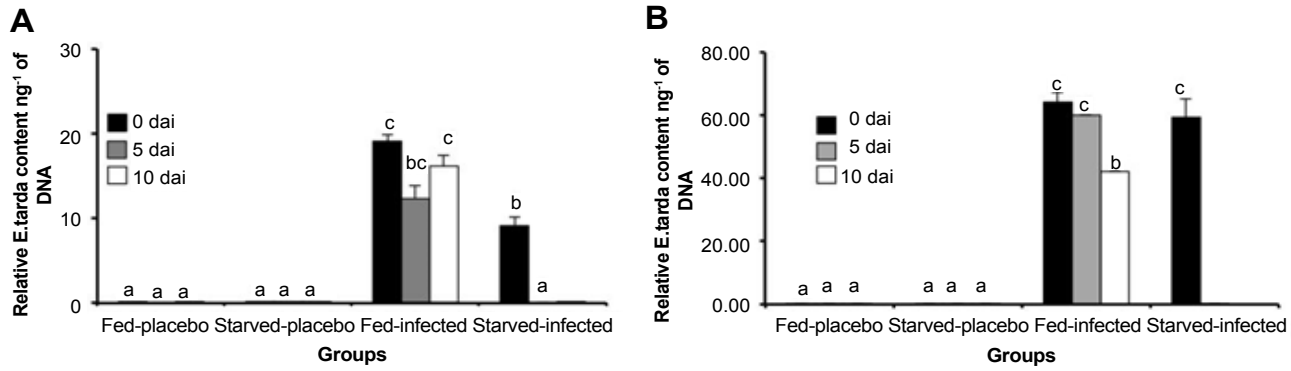

Figure 1: Real-time analysis of bacterial load in spleen (A) and muscle (B) of different experimental group. Data are shown as the mean ± S.E., and expressed as relative *E. tarda* content per ng DNA. Letters above the bars indicate that these groups differ significantly from each other at P<0.05.

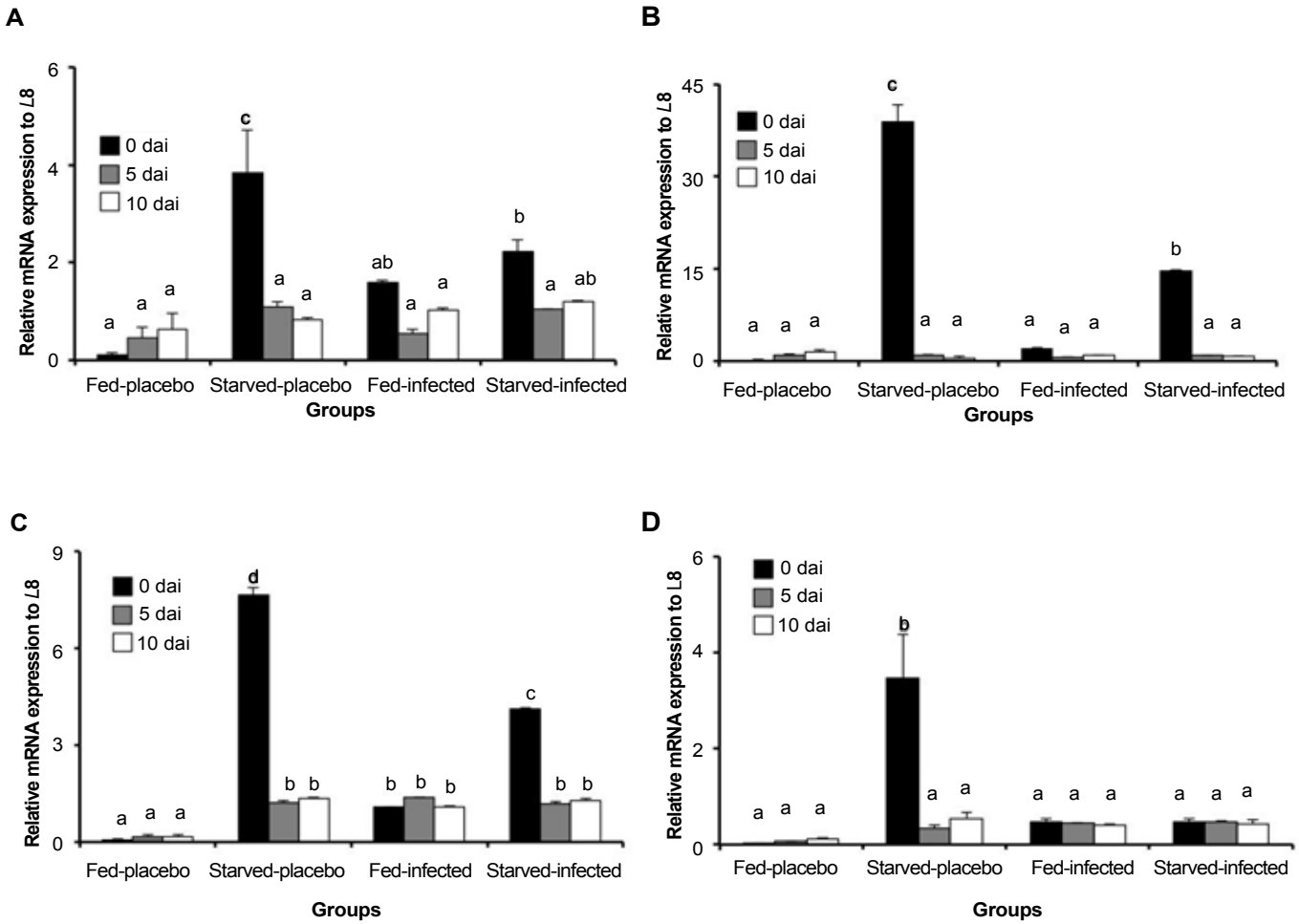

Figure 2: mRNA expression analysis of metal ion binding proteins by Real-time PCR. Changes in mRNA levels of Hepcidin1 (A), Hepcidin2 (B), Transferrin (C) and NRAMP (D) are plotted on Y-axis against different experimental groups. Data are shown as the mean ± SE, and expressed as relative abundance corrected for L8. Letters above the bars indicate that these groups differ significantly from each other at P<0.05.

Gill morphology, mucus production and oxidative stress response

Mucus, produced by mucus producing cells, is one of the first hand defence mechanism in fish. In order to find out the effect of starvation and infection on mucus production, we analysed the gill fragments by Azan staining (Figure 3). We found that, in comparison to fed-placebo group (Figures 3A and 3E), starved-placebo group showed severe hyperplasia of mucus producing cells (Figure 4). In fact, the mucus production was also enhanced in the gills of starved-placebo fish (Figures 3B and 3F). As expected, fed- infected group (Figures 3C

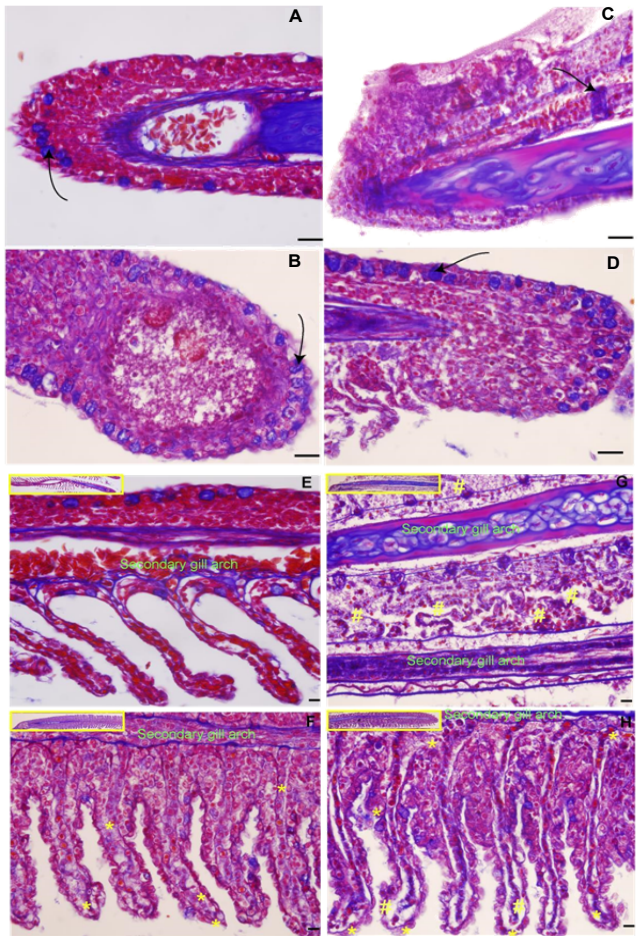

Figure 3: Effects of starvation and *E. tarda* infection on gill morphology and mucus production at 10dai. Distribution of mucus producing cells in the tip of primary gill lamellae (A-D) and Assessment of secondary gill degradation (E-H) were determined by Azan staining, where A & E, B & F, C & G, and D & H represents Fed-placebo, Starved-placebo, Fed-infected and Starved-infected groups, respectively. Note: Black arrow denotes representative mucus cells; while asterisks are given for representative degradation patterns. "#" shows severe degradation (disintegration of blood vessels and detachment of secondary gill lamellae from secondary gill arch) and "*" represents slight degradation (slight disruptions of blood vessels, breakage of the tips of the secondary gill lamellae). Mucus cell hyperplasia is observed in the starved groups as compared to the fed groups. Blue and orange color indicates the mucus and blood cells, respectively. Scale bar length - 20µm. Inset – Representative primary gill lamellae.

and 3G) showed excessive mucus production and increased blood flow along with excessive primary and secondary lamellar degradation, at 10 dai. However, after infection, the starved fish showed slight increase in mucus production than their non-infected counterpart (Figures 3D and 3H).

Both the gill SOD and CAT showed significant (P<0.05) difference between the infected and the non-infected groups, as well as between the different time points (Table 1). Higher values of gill SOD was observed in the fed-infected group than the starved-infected group.

Blood glucose and hemoglobin production

Blood glucose is a major indicator of any kind of changes in the body. In our study, blood glucose level showed no significant (P>0.05) increase after starvation at 0 and 5 dai, however reduced drastically in the 10 dai starved fish (Figure 5A). In both infected groups, decreased glucose level, in comparison to placebo groups, was observed at 0 dai, which peaked at 5 dai, and again reduced at 10 dai.

On the other hand, haemoglobin (Hb) production remained unaltered in the starved-placebo group in relation to the fed-placebo fish. However, infection reduced the Hb content significantly (P<0.05) in the fed-infected group (Figure 5B). Amongst the fed-infected and starved-infected groups, the latter had higher Hb values.

Effects on phagocytosis

At 0 dai, the starved-placebo fish showed increased NBT values, which however reduceddrastically after infection (Figure 5C). On the other hand, after infection, fed fish showed significant (P<0.05) difference in phagocytosis in comparison to the fed-placebo fish. However, we did not observe any significant (P>0.05) difference in NBT activity after infection in both the fed and starved group, except at 0 dai.

Serum profiling

No significant (P>0.05) decrease in the serum protein and globulin was observed in the starved- placebo group in comparison to the fed-placebo group (Table 2). Although the total serum protein decreased further after infection, the fed-infected group should a greater reduction than the starved- infected group. Even though albumin levels increased after infection, starvation alone was unable to change the albumin profile in red sea bream (Table 2). Similarly, the infected groups had higher A:G value than the placebo groups (Table 2).

Serum anti-oxidation characteristics

SOD and CAT activities were found to be highest in the fed-infected group followed by the starved-infected group (Table 3). The maximum difference in the activities of both the enzymes was noticed among the fed-infected and starved-infected groups at 0 dai. Although, starvation and infection had significant (P<0.05) effect on the SOD level, the CAT level remained non-significant (P>0.05) to starvation. Similarly, the GPX activity was highest in the fed-infected group, but

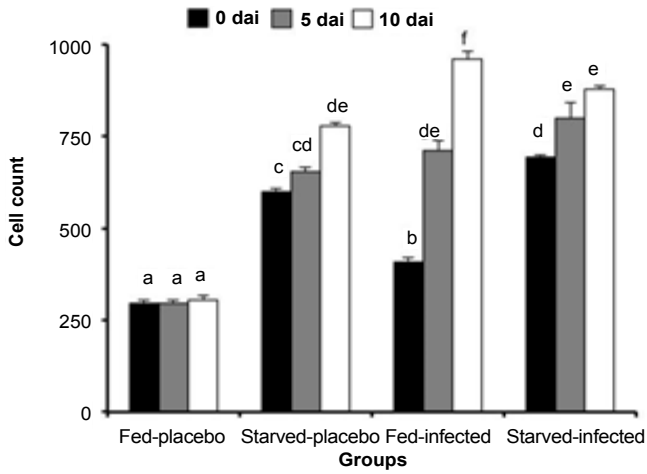

Figure 4: Analysis of the mucus-producing cell hyperplasia. The average numbers of mucus producing cells in eight randomly selected gill filaments were plotted to evaluate the mucus-producing cell proliferation potential and hence mucus production capacity of different experimental groups. Data are shown as the mean ± SE, and letters above the bars indicate that these groups differ significantly from each other at P<0.05.

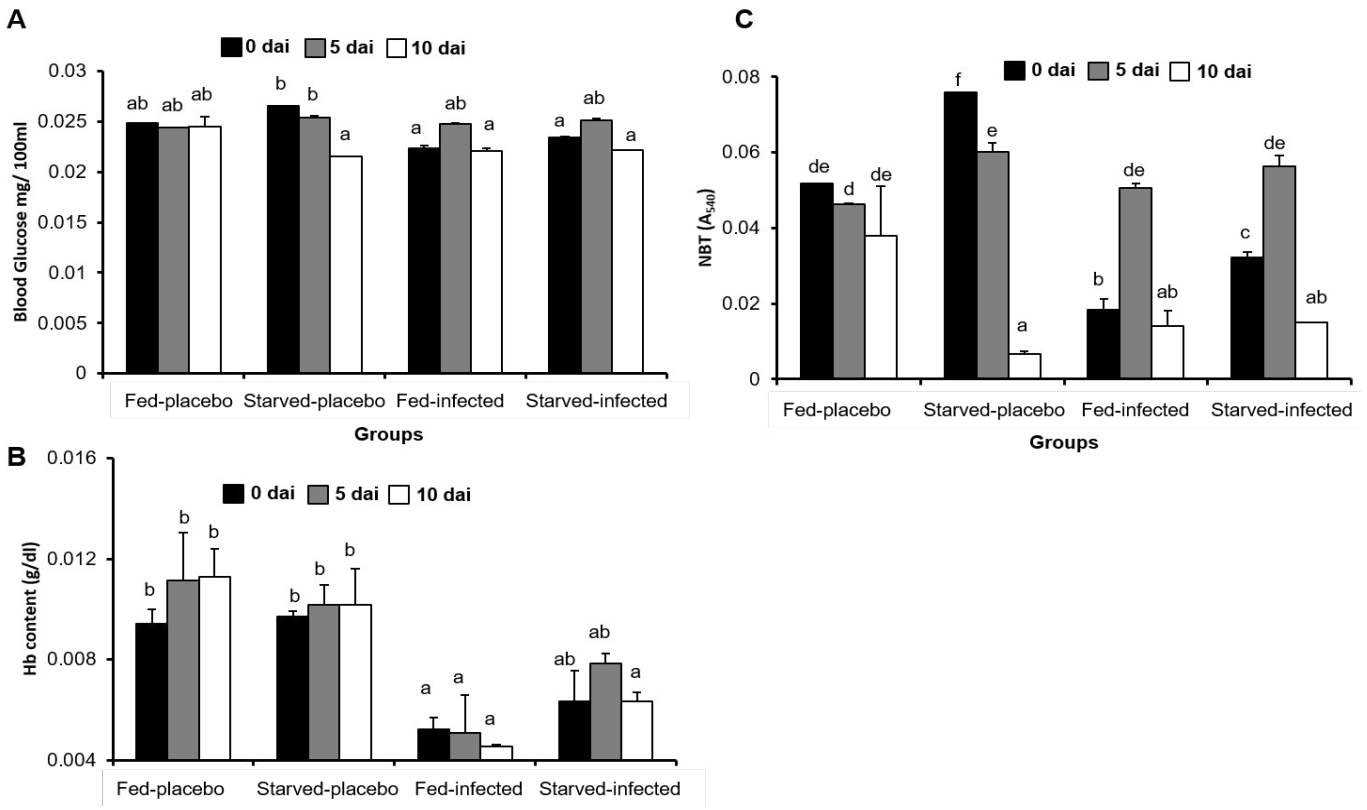

Figure 5: Biochemical analysis of blood samples from different groups. Blood glucose (A), Hemoglobin content (B) and Respiratory burst activity (NBT) (C) were measured. Data are shown as the mean ± SE, and letters above the bars indicate that these groups differ significantly from each other at P<0.05.

Parameters		Placebo			Mean Placebo	Infected			Mean Infected	Overall Mean	Significance (P<0.05) by three way ANOVA						
		T0	T5	T10		T0	T5	T10			St	T	I	StT	StI	TI	StTI
Serum protein	Fed	0.75 ± 0.01^{d}	0.53 ± 0.02^{abc}	0.77 ± 0.04^{abcd}	0.68 ± 0.02^{B}	0.39 ± 0.11^{a}	0.66 ± 0.04^{bcd}	0.51 ± 0.00^{ab}	0.52 ± 0.04^{A}	0.60 ± 0.03	NS	S	S	NS	NS	S	S
	Starved	0.60 ± 0.04^{dc}	0.64 ± 0.01^{abcd}	0.68 ± 0.04^{abc}	0.64 ± 0.02^{B}	0.44 ± 0.07^{abcd}	0.62 ± 0.04^{abc}	0.64 ± 0.06^{ab}	0.57 ± 0.04^{AB}	0.60 ± 0.02							
	Mean	0.67 ± 0.02	0.59 ± 0.02	0.73 ± 0.03	0.66 ± 0.02^{N}	0.42 ± 0.07	0.64 ± 0.03	0.57 ± 0.03	0.54 ± 0.03^{M}								
Albumin	Fed	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{AB}	0.05 ± 0.02^{b}	0.03 ± 0.01^{b}	0.01 ± 0.00^{b}	0.03 ± 0.01^{B}	0.02 ± 0.00	NS	S	S	NS	NS	S	NS
	Starved	0.02 ± 0.00^{ab}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{A}	0.06 ± 0.03^{ab}	0.03 ± 0.01^{b}	0.01 ± 0.00^{ab}	0.03 ± 0.01^{B}	0.02 ± 0.00							
	Mean	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00^{N}	0.06 ± 0.02	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.01^{M}								
Globulin	Fed	0.73 ± 0.01^{d}	0.52 ± 0.02^{abc}	0.76 ± 0.04^{abc}	0.67 ± 0.02^{B}	0.34 ± 0.11^{a}	0.64 ± 0.04^{bcd}	0.50 ± 0.00^{abc}	0.49 ± 0.04^{A}	0.58 ± 0.03	NS	S	S	NS	NS	S	S
	Starved	0.58 ± 0.04^{cd}	0.63 ± 0.01^{ab}	0.67 ± 0.04^{ab}	0.63 ± 0.02^{B}	0.38 ± 0.05^{a}	0.59 ± 0.04^{abc}	0.63 ± 0.06^{abc}	0.53 ± 0.03^{A}	0.58 ± 0.02							
	Mean	0.66 ± 0.02	0.57 ± 0.02	0.72 ± 0.03	0.65 ± 0.02^{N}	0.36 ± 0.06	0.62 ± 0.03	0.57 ± 0.03	0.51 ± 0.03^{M}								
A:G	Fed	0.02 ± 0.00^{a}	0.03 ± 0.00^{ab}	0.01 ± 0.00^{a}	0.02 ± 0.00^{A}	0.19 ± 0.06^{c}	0.05 ± 0.01^{ab}	0.01 ± 0.00^{a}	0.08 ± 0.02^{B}	0.05 ± 0.01	NS	S	S	NS	NS	S	NS
	Starved	0.03 ± 0.01^{ab}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{A}	0.13 ± 0.04^{c}	0.05 ± 0.02^{ab}	0.02 ± 0.00^{a}	0.07 ± 0.02^{B}	0.04 ± 0.01							
	Mean	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00^{N}	0.16 ± 0.04	0.05 ± 0.01	0.01 ± 0.00	0.07 ± 0.01^{M}								

Data expressed as mean ± SE (n = 4). All significance are measured at P<0.05. Different small superscript (a, b, c, d, etc.) represent the level of significance among different treatment groups at different sampling points for a particular parameter. Different capital superscript (A, B, C) represents significant difference between means of different experimental groups (fed-placebo, starved-placebo, fed-infected and starved-infected groups). Overall mean value having different capital superscript (M & N, X & Y) vary significantly. S-Significant, NS-Non-significant. Units: Serum protein – mg ml^{-1}; Albumin – mg ml^{-1}; Globulin – mg ml

Table 2: Effect of starvation (St), *E. tarda* infection (I), treatment time (T) and their interactions on serum protein profile in *P. major*.

Parameters		Placebo			Mean Placebo	Infected			Mean Infected	Overall Mean	Significance (P<0.05) by three way ANOVA						
		T0	T5	T10		T0	T5	T10			St	T	I	StT	StI	TI	StTI
SOD	Fed	1.79 ± 0.18^{a}	2.96 ± 0.30^{ab}	1.71 ± 0.10^{a}	2.15 ± 0.15^{A}	23.70 ± 6.81^{d}	2.07 ± 0.26^{ab}	2.44 ± 0.00^{ab}	9.40 ± 2.79^{C}	5.78 ± 1.45^{Y}	S	S	S	S	S	S	S
	Starved	1.97 ± 0.48^{a}	2.79 ± 0.06^{ab}	2.15 ± 0.37^{ab}	2.31 ± 0.21^{AB}	6.70 ± 1.08^{c}	2.29 ± 0.37^{ab}	0.690 ± 05^{a}	3.23 ± 0.57^{B}	2.77 ± 0.30^{X}							
	Mean	1.88 ± 0.25	2.88 ± 0.15	1.93 ± 0.19	2.23 ± 0.13^{M}	15.20 ± 3.81	2.18 ± 0.22	1.56 ± 0.18	6.31 ± 1.46^{N}								
CAT	Fed	194.84 ± 20.2^{ab}	107.38 ± 8.76^{a}	96.52 ± 12.85^{a}	132.91 ± 11.11^{A}	847.21 ± 316.84^{d}	106.13 ± 11.13^{a}	217.92 ± 0.00^{a}	390.4 ± 116.49^{B}	261.66 ± 60.07	NS	S	S	S	S	S	NS
	Starved	292.62 ± 45.0^{abc}	221.05 ± 21.5^{bc}	232.09 ± 32.72^{bc}	248.59 ± 20.02^{B}	342.39 ± 29.45^{c}	164.59 ± 16.02^{ab}	248.98 ± 21.50^{ab}	251.99 ± 17.80^{B}	250.29 ± 13.30							
	Mean	243.73 ± 26.2	164.22 ± 16.43	164.30 ± 22.26	190.75 ± 13.28^{M}	594.80 ± 164.26	135.36 ± 11.32	233.45 ± 11.00	321.20 ± 50.08^{N}								
GPX	Fed	85.21 ± 1.86^{abc}	91.58 ± 2.47^{bc}	84.49 ± 0.00^{abc}	87.09 ± 1.14^{AB}	102.86 ± 1.93^{c}	118.34 ± 21.47^{bc}	87.24 ± 1.01^{bc}	102.82 ± 7.31^{B}	94.96 ± 3.79^{Y}	S	S	NS	NS	S	NS	NS
	Starved	70.02 ± 5.52^{ab}	93.17 ± 3.17^{bc}	75.67 ± 3.01^{ab}	79.62 ± 2.82^{A}	81.45 ± 0.80^{abc}	91.00 ± 2.49^{bc}	64.38 ± 3.19^{a}	78.94 ± 2.29^{A}	79.28 ± 1.80^{X}							
	Mean	77.62 ± 3.26	92.38 ± 1.97	80.08 ± 1.73	83.36 ± 1.57^{M}	92.16 ± 2.46	104.67 ± 10.95	75.81 ± 2.89	90.88 ± 4.06^{M}								
TAC	Fed	186.17 ± 11.53^{b}	148.35 ± 4.07^{ab}	85.74 ± 0.00^{a}	140.09 ± 8.04^{B}	443.57 ± 61.2^{c}	157.48 ± 5.38^{ab}	180.52 ± 7.99^{ab}	260.52 ± 29.71^{C}	200.30 ± 16.87^{Y}	S	S	S	S	S	S	S
	Starve	117.91 ± 10.09^{a}	85.09 ± 3.70^{a}	94.93 ± 7.67^{a}	99.31 ± 4.86^{A}	182.48 ± 9.87^{ab}	166.39 ± 5.84^{ab}	145.52 ± 6.93^{ab}	164.80 ± 5.03^{B}	132.05 ± 5.21^{X}							
	Mean	152.04 ± 10.33	116.72 ± 7.12	90.33 ± 3.87	119.70 ± 5.26^{M}	313.02 ± 40.7	161.93 ± 3.99	163.02 ± 6.33	212.66 ± 16.00^{N}								
SMC	Fed	20.56 ± 3.48^{b}	28.07 ± 2.93^{b}	16.31 ± 0.00^{a}	21.65 ± 1.69^{A}	22.51 ± 1.26^{b}	31.02 ± 2.73^{bc}	22.62 ± 0.13^{b}	25.38 ± 1.18^{B}	23.51 ± 1.05	NS	S	NS	S	NS	S	NS
	Starved	31.15 ± 1.5^{c}	24.30 ± 2.13^{b}	13.42 ± 2.49^{a}	22.951 ± 70^{AB}	23.57 ± 3.82^{b}	21.69 ± 0.53^{b}	20.67 ± 1.71^{b}	21.98 ± 1.38^{B}	22.47 ± 1.09							
	Mean	25.85 ± 2.16	26.18 ± 1.81	14.87 ± 1.26	22.30 ± 1.19^{M}	23.04 ± 1.97	26.36 ± 1.67	21.64 ± 0.86	23.68 ± 0.93^{M}								
PAC	Fed	165.62 ± 8.48^{ab}	120.28 ± 4.36^{ab}	69.43 ± 0.00^{a}	118.44 ± 7.32^{B}	421.05 ± 61.2^{c}	126.46 ± 6.90^{ab}	157.91 ± 7.93^{ab}	235.14 ± 30.03^{C}	176.79 ± 16.84^{Y}	S	S	S	S	S	S	S
	Starved	86.77 ± 10.25^{a}	60.79 ± 2.39^{a}	81.51 ± 7.37^{a}	76.36 ± 4.57^{A}	158.91 ± 12.7^{ab}	144.70 ± 6.21^{ab}	124.85 ± 7.82^{ab}	142.82 ± 5.75^{B}	109.59 ± 5.37^{X}							
	Mean	126.19 ± 10.48	90.53 ± 6.66	75.47 ± 3.82	97.40 ± 4.96^{M}	289.98 ± 41.0	135.58 ± 4.92	141.38 ± 6.45	188.98 ± 16.14^{N}								

Data expressed as mean ± SE (n = 4). All significance are measured at P<0.05. Different small superscript (a, b, c, d, etc.) represent the level of significance among different treatment groups at different sampling points for a particular parameter. Different capital superscript (A, B, C) represents significant difference between means of different experimental groups (fed-placebo, starved-placebo, fed-infected and starved-infected groups). Overall mean value having different capital superscript (M & N, X & Y) vary significantly. S-Significant, NS-Non-significant. Units: Superoxide dismutase (SOD) – units mg protein^{-1}; Catalase (CAT) – units mg protein^{-1}; Glutathione peroxidase (GPX) – nmol min^{-1} ml^{-1}; Total anti-oxidation capacity (TAC) – mM trolox equivalent; Small molecule anti-oxidation capacity (SMC) - mM trolox equivalent; Protein anti-oxidation capacity (PAC) – mM trolox equivalent^{-1}

Table 3: Effect of starvation (St), *E. tarda* infection (I), treatment time (T) and their interactions on serum stress enzymes activity in *P. major*.

reduced significantly (P<0.05) in the starved-infected group (Table 3). Starvation and infection interactively had a significant effect (P<0.05) on the GPX activity.

Similar to other antioxidant markers, SMC and PAC values were significantly (P<0.05) altered after infection (Table 3). The TAC values showed significant increase in the fed-infected groups as compared to the starved-infected groups (Table 3).

Transcriptional changes

In spleen, except for 10 dai, *TNFα* levels did not differ among the various experimental groups (Figure 6A). At 10 dai, starved group samples had higher *TNFα* expression than its fed counterparts. Although similar results were observed for *IL-1β*, the differences were more pertinent (Figure 6B). *MHCIIA* expressions were gradually up-regulated in the starved groups at all-time points (Figure 6C). In case of chemokines, starvation induced spleenic *IL8* expression in a time dependent manner, which upon infection increased more steeply (Figure 6D). However, the fed-infected group showed sudden increase in *IL8* expression and later became static at 5 and 10 dai.

Programmed cell death analysis in gill lamellae

In order to analyses the starvation associated programmed cell death in the gill lamellae, we performed two-colored immunohistochemistry of the gills, using Annexin V (apoptosis cell death marker) and Beclin1 (autophagic cell death marker) antibodies. The fed-placebo (Figure 7A) group showed comparatively lesser Annexin V stained cells than the starved-placebo (Figure 7B) group. The fed- infected (Figure 7C) group showed numerous Annexin stained cells. The starved-placebo group had a moderate amount of Annexin V/Beclin1 double stained cells, which however, increased multi-fold in the starved-infected (Figure 7D) group.

Evaluation of disease resistance capability

When compared to fed-placebo group, survivability of both starved groups didn't show any significant difference at 0 and 5 dai, however, slightly reduced at 10 dai (Figure 8). On the other hand, survivability of fed-infected group decreased gradually at different time points. Evidently, the starved- infected group showed better survivability than the fed-infected group.

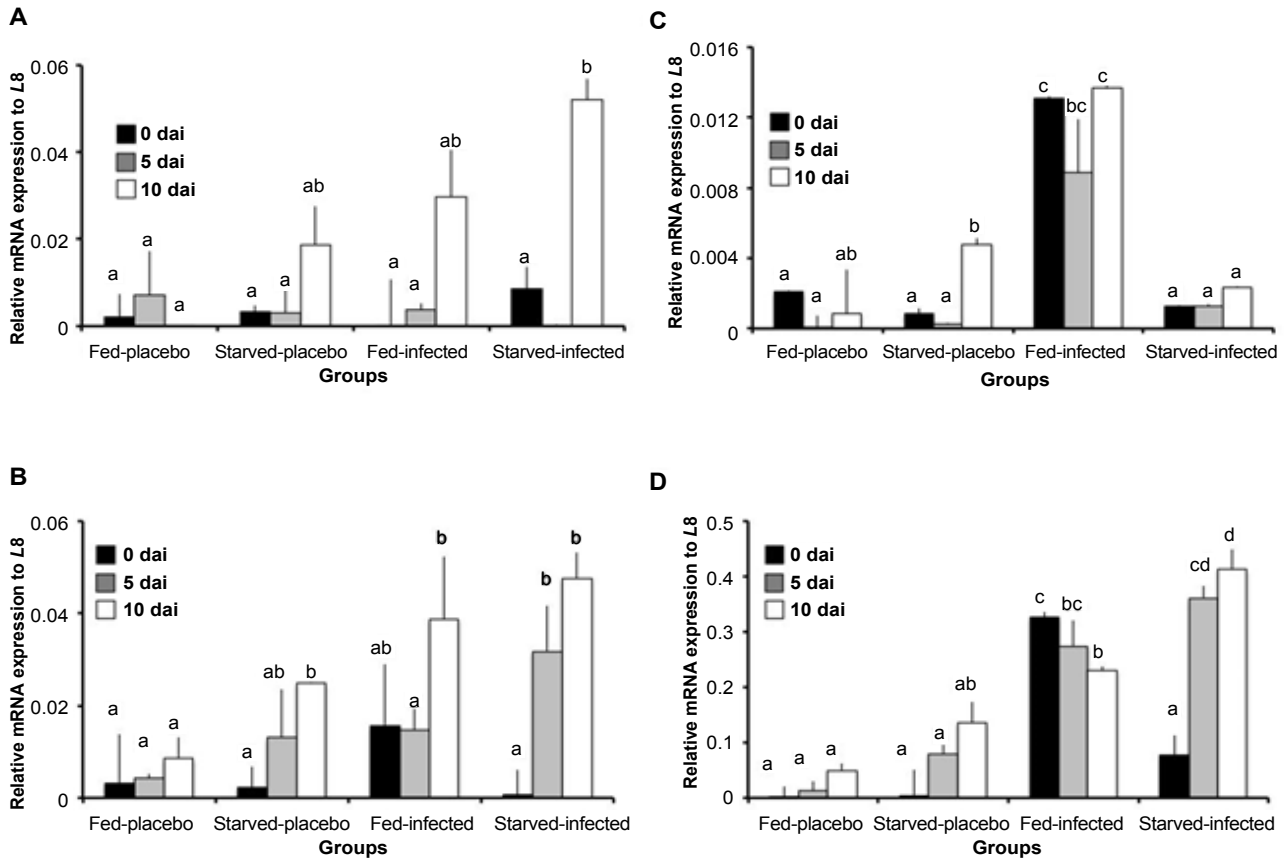

Figure 6: mRNA expression analysis of cytokines by Real-time PCR. Changes in mRNA level of TNFα (A), IL-1β (B), MHCIIA (C) and IL8 (D) are plotted on Y-axis against different experimental groups. Data are shown as the mean ± SE, and expressed as relative abundance corrected for L8. Letters above the bars indicate that these groups differ significantly from each other at P<0.05.

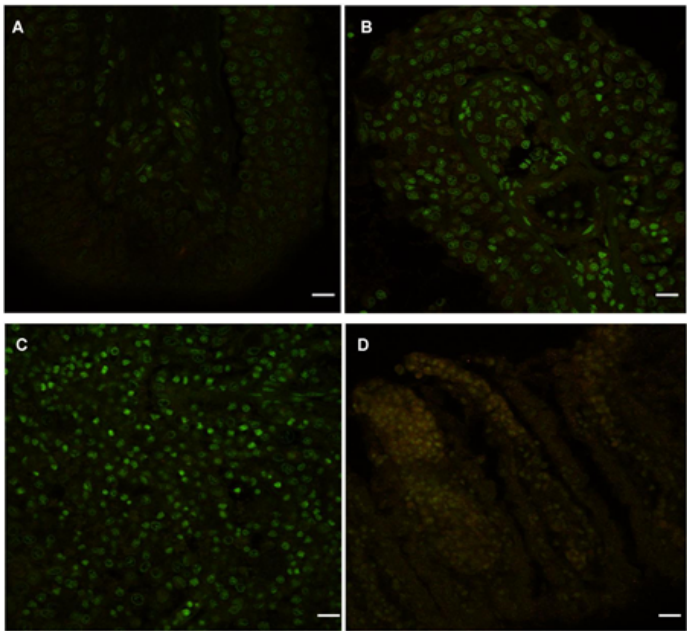

Figure 7: Starvation and infection associated programmed cell death in the gill lamellae. Two-colored immunohistochemical analysis, using Annexin V (green) and Beclin1 (red), was done to evaluate the programmed cell death in the gill lamellaes of Fed-placebo (A), Starved-placebo (B), Fed-infected group (C), and Starved-infected (D) groups at 10 dai. Bar length – 20 µm.

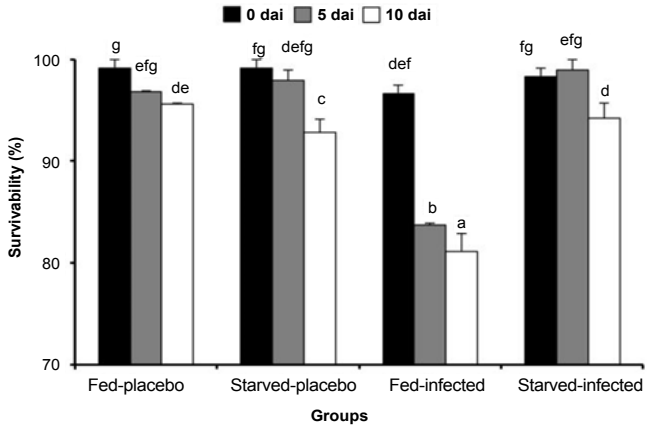

Figure 8: Evaluation of survivability of different experimental groups. Data are shown as the mean ± SE, and letters above the bars indicate that these groups differ significantly from each other at P<0.05.

A descriptive statistical analysis was made to obtain a global idea about the DRI and its variability between groups. The mean, the standard deviation, the minimum and the maximum values of the DRI for each grouping criteria are represented in Table 4. Based on the comparison of the means, we found that the fed-infected fish, followed by starved-infected and starved-placebo fish had the lowest average disease resistance. As expected, the fed-placebo group showed the best average resistance. More detailed explanation about the statistical evaluation of DRI and the effects of infection (Supplementary Table 2) and starvation (Supplementary Table 3) on overall fish condition are given in the supplementary information (Supplementary Information 1).

Discussion

Starvation, during an immune-compromised situation, has been known to be an effective management strategy in curbing the infection-related mortality in numerous organisms [11,14]. In the present study, short-term starvation resulted in a drastic reduction in the *E. tarda* content in both spleen and muscle, along with the increase in several iron homeostasis related genes. We also recorded positive alterations in the blood and serum parameters, cytokine production, mucus cell number and gill morphology in the starved fish. Moreover, we observed that, upon *E. tarda* infection, the starved fish exhibited enhanced survivability in comparison to the fed counterparts. Thus, the present study highlights the importance of short-term starvation in helping the animal to mount an immunological response during *E. tarda* infection, and successfully fighting against the disease.

Bacteria generally use the host's circulating free metal ions to propagate inside the host [22,23]. Nutrient, especially iron, withholding is an important host defense mechanism during bacterial infection, which suppresses bacterial outgrowth [22]. The major regulators of iron homeostasis i.e. *Hepcidin, Transferrin, Ferritin*, etc., are influenced by both starvation and infection. Recently, Lin et al. [23] showed that addition of *Transferrin* could improve the health of infected mice probably by disrupting the microbial transmembrane potential and sequestering the free nutrient iron content in the body [22,24]. Similarly, we also observed a significant (P<0.05) increase in *Transferrin* expression in the spleenic cells of starved-infected fish. It is noteworthy to mention that, *Transferrin* only binds and transports the free iron but *Hepcidin* helps to store the iron inside different cells and tissue, which reduces the circulating concentration of iron in the serum [25]. Similar to *transferrin*, we also observed increased Hepcidin 1 and 2 mRNA expressions in starved-infected fish than the fed-infected groups, which may indicate a conserved iron sequestering mechanism, prevalent in fish, that in-turn helps to fight against bacterial infections. These high levels of iron regulatory genes in the starved groups highlights the tug-of-war over iron between the host and the invading bacteria [26], and further aids in impeding the growth of the pathogenic bacteria due to reduced iron concentration in the host body.

Iron is also an essential component for erythropoiesis [27], oxygen transport (via hemoglobin) [26], bacterial sustainability [22] and other cellular mechanisms. Starvation is instrumental in reducing the circulating iron in the body by increasing the tissue iron retention rate [28]. Free circulating iron content and hemoglobin concentration are correlated in various organisms [29]. According to Lim and Klesius [30], four weeks starved channel catfish exhibited significant reduction in the hemoglobin content. However, we found no significant difference in the hemoglobin content between the fed and starved-placebo groups. Similar to our data in red sea bream, many other research groups also reported that prolonged starvation did not alter the hemoglobin content in other fish species, eg. Olive flounder *(Paralichthys olivaceus)* [31], Red progy *(Pagrus pagrus)* [32]. Unlike starvation, infection is a ubiquitously known factor for reducing hemoglobin content in various fish species [33]. In our present work, upon infection, the hemoglobin content was significantly (P<0.05) reduced in both the starved and fed group, which might suggest an alternative mode of hemoglobin reduction, via reduced availability of certain amino acid and protein in fish [34]. Mohapatra et al. [17] also reportedsignificantly low hemoglobin content in the *Aeromonas hydrophila* infected *Labeo rohita* fingerlings than the non-infected fish.

Starvation is known to alter the blood glucose concentration, depending on the duration of starvation, interspecies variability, age, and others [35-37]. Several fish species either utilize their stored liver glycogen for the maintenance of metabolic functions during short term starvation [38,39] or reduce the rate of glucose utilization by increasing the gluconeogenesis process [39,40]. In our present study, we recorded no remarkable changes in the plasma glucose concentrations among all the different experimental groups at 0 and 5 dai, but a slight reduction was noticed in the 10 dai starved groups. This reduction in the blood

Grouping criteria	Treatment groups	Mean	Standard deviation	Min	Max
0dai	Placebo	60.09	3.56	56.28	67
	Infected	47.99	5.86	37.23	55.48
	Total	54.04	7.81	37.23	67
5dai	Placebo	52.71	2.81	48.97	56.53
	Infected	52.32	2.24	49.91	56.09
	Total	52.51	2.46	48.97	56.53
10dai	Placebo	49.1	3.78	45.02	55.02
	Infected	46.71	2.16	42.62	49.09
	Total	47.91	3.22	42.62	55.02
Starved	Placebo	51.85	5.7	45.02	60.14
	Infected	50.1	3.06	47.14	55.48
	Total	50.98	4.56	45.02	60.14
Fed	Placebo	56.08	5.06	49	67
	Infected	47.91	5.34	37.23	56.09
	Total	51.99	6.58	37.23	67

Table 4: Descriptive statistical analysis of the effect of treatment time and food on overall disease resistance index (DRI).

glucose level might indicate that the rate of glucose production bygluconeogenesis was not sufficient to negate the effect of starvation for a longer period of time [39].

Serum profiling is essential in assessing the physiological status of any animal. Any change in the serum condition is reflective of the physiological, nutritional and pathological condition of the animal. The reduced serum protein level and the simultaneous increase in the A:G in the fed-infected group points towards the stressful condition of the fish due to bacterial infection [41,42]. Oxidative stress eventuates when the rate of production of ROS (reactive oxygen species) surpasses the scavenging capacity of the antioxidants [43], resulting in the damage of several cellular components [44]. Several anti-oxidant enzymes such as SOD, which curb the potentially toxic superoxide radical, CAT and GPX, which hydrolyse and controls the H_2O_2 in the body, are responsible in ameliorating the stress response of the animal [45]. Bacterial infection is known to compromise the immune system of the fish [17], mostly by increasing the ROS production leading to severe oxidative stress. In this study, we observed higher levels of all the three stress enzymes (SOD, CAT, GPX) in the fed-infected groups, which is indicative of higher oxidative stress level that could have resulted due to the *E. tarda* infection [46]. The decreased levels in the SOD and CAT levels in the starved-infected groups could correlated with increased resistance to oxidative stress due to the effect of prior starvation [47]. Although we had expected a more pronounced SOD and CAT level in the gills, since it the primary contact site for water borne pathogenic exposure, very low levels of these enzymes were observed in comparison to the serum. This could indicate that the pro-oxidation capacity is quite low in the gills than the other organs [43].

Antioxidants play a vital role in preventing the formation of and scavenging of free radicals and other potentially toxic oxidizing species. The total antioxidant capacity (TAC) of fish acts as a reliable biomarker for stress as it exhibits the metabolic capacity of both the antioxidant enzymatic and non- enzymatic system to external stimuli [48,49]. We observed a significant increase in the TAC value in the fed-infected group, which however decreased substantially in the starved-infected group. In the present investigation, *E. tarda* infection might be responsible for the increase in the oxidative stress which could have led to the up-regulation antioxidants in the fish [50].

As we have discussed earlier, starvation might be an integral part of immune enhancement of fish, probably by the improvement of innate immunity. The essentiality of cell-mediated immunity in the host defense against bacterial infection is well documented in mammals [51,52]. In fish, upon pathogenic infection, pro-inflammatory cytokines *(IL-1β and TNFα),* secreted by the activated immune- related cells (macrophages, neutrophil, lymphocytes etc), results in eliciting major immune responses [53,54]. TNFα is also responsible for various other host responses, including cell proliferation, differentiation, apoptosis, necrosis, and induction of other cytokines [55]. In the present study, up- regulated expression of the pro-inflammatory cytokines was recorded both after starvation and infection; suggesting the action of various inflammatory stimuli, working alone or in combination, to induce such response. Several studies with zebra fish *(Danio rerio),* carp, etc., have showed that experimental infection with *E. tarda* resulted in differential acute alteration of the inflammatory cytokines, *IL-1β* and *TNFα* [56,57]. Similar increased immune responses, especially *IL-1β* expression, were reported in Atlantic char when the starved fish were treated with lipopolysaccharides (LPS) [58]. This increased immune response might have caused an energy demanding condition, thereby resulting in a unique metabolic alteration in order to deal with the extreme stress without eliciting immune suppression [58]. Since a brief bacterial challenge resulted in gradual up-regulation of these cytokines, it can be considered that they follow a similar activation mechanism probably via endothelial cell mediated or cortisol induced cytokine production [59,60]. Cytokines and chemokines are the major innate immune response mediators and their expression is the key marker of immune function [61]. *IL8*, an important chemokine, is excessively produced in response to several stimuli (LPS or Poly I:C). In present study, similar elevated *IL8* and *MHCIIA* levels were observed in the infected groups, suggesting that chemokines play a significant role in the immune boosting or host innate defense after *E. tarda* infection.

Similar to mammals and other teleosts, our results also demonstrated the importance of both cell-mediated and innate immunity in safeguarding against intracellular bacterial infection [52]. In the present investigation, all the studied immune-related genes, although showed an increasing trend along with days of starvation, no significant difference was observed between the fed and starved fish, which could suggest that these animals were capable of withstanding starvation related stress conditions (especially the energy demand) without hampering the immune status [58]. However, after infection, majority of the genes were significantly ($P<0.05$) increased in the starved fish than their fed counterparts. A plausible explanation for this increase could be that the host defense mechanism was initiated and the levels of cytokines were raised to a higher level in starved fish, which gave them an upper-hand in terms of immune enhancement. Similar observations were recorded regarding the DRI, where the starved-infected group had better response than the fed-infected ones, suggesting that some unknown mechanism are at work in the starved fish which facilitates in the immune status fish.

Gill is one of the major doorways for pathogenic entry in fish owing to its direct exposure to the external environment [17,62]. Mucus secretion from epithelial cells of the gill is considered to the first hand response against any kind of stress or infection. Mucus contains many active components including proteases, antimicrobial peptides, lysozymes, which are long known to protect the organism from external bacterial or parasitic invasion [63,64]. Our gill histological observations depicted maximum gill lamella degradation and blood vessels deterioration in the fed-infected fish at 10 dai, which could be indicative of the harmful effect of *E. tarda* infection. However, the starved-infected fish portrayed slightly lesser gill damage than its fed partners, accentuating the beneficial role of starvation during infection. This improved condition of the animal might be associated with several factors (i) mucus production was increased beforehand, due to starvation which creates a physical barrier for direct bacterial invasion in the starved- infected fish [63] (ii) improved oxidative stress response and antioxidants profile might have alleviated the cells from bacterial induced stress (iii) Starvation could have resulted in activation of certain autophagic reaction which in turn protected the fish against the harmful pathogenic bacteria [47,65].

Conclusion

In relation to this, we also observed better survivability in the starved-infected groups than fed- infected ones. Interestingly, DRI also showed a similar trend with the survivability of fish, which although decreased in starved groups (compared to fed-placebo) but remained significantly higher than fed-infected ones. This suggests that starvation, even though undesirable for a lengthy period of time, can be a boon for preventing infection in fish. Additionally, DRI could be a new global index for assessing the overall fish health.

The data presented in this study clearly shows that starvation is beneficial in controlling bacterial invasion in red sea bream. Although our data suggests that multiple pathways i.e. free iron regulation, antioxidant production, oxidative stress response, phagocytosis, cytokine and chemokine production, autophagic induction are important for total resistance, the inter-relating factors/mechanisms needs further investigation to get a comprehensive idea about starvation induced disease resistance mechanism in fish.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Author Contributions

Conceived and designed the experiments: SM TC. Performed the experiments: SM TC. Analyzed the data: SM TC RHK. Contributed reagents/materials/analysis tools: SS TM KO. Wrote the paper: SM TC.

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