ANTIOXIDANT RELATED GENE EXPRESSION PROFILE OF MOLLYFISH (*POECILIA SPHENOPS*) INFECTED WITH *AEROMONAS HYDROPHILA*

A Dissertation Submitted to st. teresa's college (autonomous), ernakulam in Partial Fulfilment of the Requirement
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SUBMITTED BY

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CERTIFICATE

This is to certify that the dissertation entitled "**Antioxidant Related gene expression profile of Molly fish (*Poecilia sphenops*) infected with *Aeromonas hydrophila***" submitted to St. Teresa's College (Autonomous), Ernakulum, in partial fulfillment of the requirement of award of degree of Master of Science in Zoology is an authentic work carried out by **Ms. Rosna Sajeev** (SM21ZOO011) in the academic year 2021 - 2023 under the guidance and supervision of **Dr. Beena P S, Ph.D.** (External Guide) Director of OmicsGen LifeSciences Pvt. Ltd, Kakkanad, Kochi and **Dr. Damaris Benny Daniel** (Internal Guide), Assistant Professor, Department of Zoology, St. Teresa's College, Ernakulum.

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ABSTRACT

An opportunistic bacterium known as *Aeromonas hydrophila* has been associated with a variety of fish disease outbreaks. Disease due to *A. hydrophila* affects aquaculture, one of the industries with the fastest growth and a significant source of high-quality protein. Unfortunately, disease outbreaks limit aquaculture production, which has an impact on the nation's economic growth and the socio-economic status of the population that completely depends on this sector.

This study was conducted on *Poicelia sphenops* which is a representative organism taken to investigate the magnitude of gene expression of catalase (CAT), superoxide dismutase (SOD), Glutathione peroxidase (GPX), Glutathione S Transferase (GST), and heat shock protein 70 (HSP70) due to the *A. hydophila* infection. The fish along with the control were treated with a trace amount of bacteria (*A. hydrophila)* and after about forty-five hours the tissues were isolated from both the treated and control. RNAs were isolated and the real-time polymerase chain reaction (RT-PCR) was conducted to observe the change in the magnitude of gene expression along with the housekeeping gene (GAPDH) for normalization. A significant change in the expression of catalase (CAT) and superoxide dismutase (SOD) was observed. That is, the catalase gene (CAT) showed a significant up regulation, whereas superoxide dismutase (SOD) was slightly down regulated. All of these findings imply that the innate immune system was significantly altered during an infection to provide the primary defence for the *P. sphenops*.

INTRODUCTION

Fish provides high-quality protein for human consumption, and aquaculture is one of the world's most effective methods of producing high-quality protein. It is the most rapidly expanding food supply sector, and it contributes to food security. Aquaculture can contribute greatly to and protect worldwide food supplies when produced in environmentally and socially responsible ways. In contrast to farming land animals such as pigs and cows, a fishery is among the most resourceful and environmentally friendly methods of producing protein for human consumption. Aquaculture also benefits people and communities worldwide by offering decent jobs and business opportunities, as well as a source of income for farmers and revenue for governments.

Freshwater aquaculture accounts for more than 95% of all aquaculture production in India, where it has increased more than six and a half times in the past two decades. The primary contribution to India's position as the third-largest aquaculture producer in the world comes from freshwater aquaculture, whose percentage of inland fisheries has increased from 46% in the 1980s to over 85% in recent years. From 0.37 million tons in 1980 to 4.03 million tonnes in 2010, freshwater aquaculture had an astonishing ten-fold growth, with a mean yearly increase of above 6% (Ayyappan *et al*., 2001;Michieal *et al*., 2017).

Nowadays, various environmental stresses such as the influence of pathogenic microorganisms, chemical exposure, extreme weather changes, and water and air pollution can affect different organisms in several ways. In extensive aquaculture, all these stresses lead to adverse effects on fish that lead to suppression of the immune system, and eventually, the fish become vulnerable to various infectious diseases.

The Gram-negative *A. hydrophila* bacteria of the Aeromonadaceae family are in charge of a wide variety of challenges in diverse kinds of farmed fish. Over the years innumerable laboratory-based attempts have been made to develop vaccines against this pathogen (Nayak, 2020). Fish health is frequently threatened by *Aeromonas*, especially in aquaculture settings where vigorous feeding is done. There are three species of movable *Aeromonas*: *A. hydrophila*, *A. caviae*, and *A. sobria*. Although they are often saprophytic, they could be harmful to fish. Many fish diseases, such as aeromonad septicemia and

erythrodermatitis are caused as a result of pathogenic aeromonads (MAS). Direct contact with diseased fish or environmental pollution can be the reason for the spread of the infection (Zmys1owska *et al*., 2008). Infection due to this particular variety of pathogens can cause several diseases, even sudden death. The survival ability of fish when inoculated with a trace amount of bacteria (*A. hydrophila*) gives detailed information about the immune factors that are involved during the bacterial infection.

One of the fishing operations that are expanding most quickly is aquaculture. Fish farming can offer the owner various advantages along with the rising market demand. Many challenges can arise throughout this exercise at any time. Management with quality is one of the major challenges in fish farming. Microbes like bacteria can adversely affect fish due to poor management. The bacteria *A. hydrophyla* is one of the pathogens that affect farmed fish. A bacterium called *A. hydrophila* is detrimental to freshwater fish rearing. These bacteria can adversely affect fish of different sizes, which can result in mortality rates of up to 80%. Red spot illness or Motile Aeromonad Septicaemia (MAS) is brought on by these bacteria. These bacteria create illness epidemics with a fatality rate of 80% to 100% in 2 weeks when they infect a variety of freshwater species, including fishes like goldfish catfish, etc... Different parts of the body like the liver, Gills, kidneys, skin, and the digestive tract all contain these bacteria (Amatulloh *et al*., 2021).

There are various pathogens infecting culture fishes like Rohu, Tilapia, Carp, etc… The advancement in genetic engineering methods has made it possible to cultivate tilapia in freshwater, brackish, and marine environments. This fish is also more prolific, adaptable, and grows more quickly in the tropics than in the subtropics (Yue *et al*., 2016). Intensive culture systems are required due to the rising demand for animal protein, but these systems could raise the risk of disease due to poor water quality, higher population densities, and sudden shifts in culture conditions, which cause increased stress and lead to a variety of agent causes of diseases, mostly from streptococcal bacteria (Nakharuthai *et al*., 2016). It has been determined that the major reason for death in tilapia cultivation over the world is *Streptococcus agalactiae* (Delannoy *et al.,* l2016; Kannika *et al*., 2017). This pathogen affects failure and has caused very significant financial losses in the tilapia aquaculture business in various nations, notably Brazil (Mian *et al*., 2009; Tavares *et al.,* 2016).

The fish farming sector needs to be developed further due to the rising market demand. Nevertheless, parasitic, bacterial, and viral infections will always generate significant financial losses for the fish breeding sector. *A. hydrophila* is an important disease-causing pathogen among fishes (Chen *et al*., 2018). In recent years, research on fish immune systems and fish's reactions to pathogenic bacteria has grown significantly. Nevertheless, there isn't any information available regarding *Puntius sarana's* immune system or health. A Gram-negative bacterial infection called *A. hydrophila* causes hemorrhagic septicemia and ulcerative syndrome in freshwater fish, especially Indian carps, which results in high deaths in hatcheries and culture systems and significant financial loss (Sahoo *et al*., 2008).
Stressors expose the fish in aquaculture facilities to disease hazards. Worldwide, diseases result in significant annual losses in aquaculture, and antibiotic therapy drives up costs for aquaculture farms. Moreover, antibiotic therapy raises serious concerns about environmental contamination and the emergence of novel antibiotic-resistant bacteria. (Alderman and Hastings, 1998).

Reactive oxygen species must be managed by all aerobic organisms. They are oxygen-containing chemically reactive molecules. They develop as a by-product of oxygen's normal metabolism naturally and play crucial functions in cellular responses and homeostasis (Cadenas, 1989). Anyway, Environmental stress can cause ROS levels to spike rapidly. (Gerschman *et al*., 1954; Cadenas, 1989). Oxidative stress occurs when the organism's ability to handle the generation and deposit of ROS is exceeded. Lipids, proteins, and deoxyribonucleic acid may all be harmed by this. Certain ROS can start the self-replicating process of lipid peroxidation, which results in the formation of a peroxyl radical when a ROS is reactive enough to remove a hydrogen atom out of an intact lipid. The most common causes of cell injury are the interaction of ROS with lipids (Halliwell and Gutteridge, 1999). An inevitable part of an aerobic lifestyle is oxidative stress. Reactive oxygen species (ROS) generation and antioxidant defences in living things are out of balance, which causes it. s (Nishida, 2011).

In the majority of physiological conditions, antioxidant responses closely reflect ROS generation. The antioxidant response includes enzymatic antioxidants such as superoxide dismutase, catalase, and peroxidases (Lesser, 2006). By using several kinds of antioxidant enzymes to scavenge, neutralize, and/or detoxify ROS, organisms create defence mechanisms

to defend themselves from "oxidative stress". A crucial antioxidant enzyme known as superoxide dismutase (SOD) serves as the first line of defence for antioxidant mechanisms against ROS. It transforms O2 into less harmful H2O2, which is then transformed into H2O by the catalytic operations of catalase, a different significant antioxidant enzyme (CAT) (Mruk *et al*., 2002). Research mostly from the 1980s shows that the fish have developed antioxidant defences against the impact of reactive oxygen species. Most fish species examined thus far have been shown to include specialized enzymes, including catalase (CAT), superoxide dismutase (SOD), and enzymes dependent on glutathione (glutathione peroxidase, GPX, and glutathione reductase, (GR)) (Rudneva, 1997).

After exposure to hyperoxia, differences in SOD, CAT, or GSH-Px were discovered in the liver of Atlantic salmon. As conventional oxidative stress markers, the blood concentrations of total glutathione (GSH), oxidised glutathione (GSSG), and the consequent oxidative stress index (OSI) were measured (Olsvik *et al*., 2005). The primary antioxidant enzymes and significant oxidative stress markers are glutathione peroxidase (GPx), glutathione-s-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and glutathione-peroxidase (SOD). In non-enzymatic antioxidant defence, reduced glutathione (GSH) and oxidized glutathione disulphide (GSSG) are crucial components. Metal-binding proteins with specific roles in the detoxification of hazardous metals include ferritin, ceruloplasmin, and metallothioneins (MTs), which also have a role in the metabolism and homeostasis of important metals (Kelly *et al*., 1998).

The influence of any bacterial stress could lead to the overproduction of reactive oxygen species (ROS) (Ellis, 1998), which results in oxidative stress in fish. Certainly, an increase in the level of ROS could lead to lipid peroxidation, which likely damages cellular molecules (Sanchez *et al*., 2005).To minimize the negative impact of ROS fish use several defense mechanisms that are either enzymatic or non-enzymatic (Zaleski *et al*., 2014). Catalase is an antioxidant enzyme that is part of the enzymatic mechanisms involved in detoxification by breaking down toxic reactive oxygen species (ROS) (Sm *et al*., 2015). The rates of these enzymes, which include SOD, CAT, GSH-Px, GSH-S-transferase, and GSH reductase, have been used to measure the level of oxidative stress in cells (Oost *et al*., 2003). Even though there are many studies conducted on bacterial stresses in fish, studies on CAT gene expression are bounded. By utilizing the real-time polymerase chain reaction (PCR), it can explain the effect of bacterial stress on catalase (CAT), superoxide dismutase (SOD),

Glutathione peroxidase (GPX), Glutathione S Transferase (GST), and heat shock protein 70 (HSP70) gene expression in *P. sphenops*.

AIM

The present study aims to understand the stress tolerance in fishes by using antioxidant genes after the infection with *A. hydrophila*.

OBJECTIVE

- To identify the bacterial stress tolerance in fishes by using antioxidant gene expression.
- To evaluate the effect of bacterial infection and the magnitude of gene expression change in a selected fish species.

RELEVANCE OF THE STUDY

Aquaculture is one of the world's most effective methods of producing high-quality protein. It is the most rapidly expanding food supply sector, and it contributes to food security. It also benefits people and communities worldwide by offering decent jobs and business opportunities, as well as a source of income for farmers and revenue for governments. The pathogenic attack could lead to suppression of the immune system of cultured fishes and can cause severe infection and even death. Thus have a negative influence on aquaculture in turn also affects the economy of the country. Pathogenic outbreaks are more these days, especially, since *A. hydrophila* is a major disease-causing pathogen in fishes, which makes the study more relevant. The study on the activity of the immune response gene will give a baseline knowledge regarding the influence of *A. hydrophila* on the inherent defence system in *P. sphenops* and aquaculture fishes. The gene expression of biomarkers elucidated in this study can be used to determine the efficacy of remedial treatments or even vaccine development against this infection in future studies.

REVIEW OF LITERATURE

There are many works that conducted regarding *A. hydrophila* infection in various fishes, which includes, investigation of the specific alterations in the innate immune responses in *P.sarana* after infection with *A. hydrophila*. Early infection periods were marked by a large rise in serum myeloperoxidase, ceruloplasmin activities, and total leucocyte count as well as a drop in alternative complement activity. After infection, there was a discernible drop in blood's packed cell volume, hemoglobin content, total erythrocyte count, and plasma glucose levels. In the liver, survivors had a noticeable up-regulation of C3 and transferrin and a down-regulation of lysozyme G, interleukin 8, MnSOD, and B2M. On the other hand, it was shown that C3 was dramatically down-regulated in the kidney whereas lysozyme G, lysozyme C, interleukin 1, interleukin 8, CXCa, and MnSOD were all significantly up-regulated. The evidence points to a highly regulated innate immune system that provided *P. sarana* with protection during the infection process (Das *et al*., 2011).

A. hydrophila, an opportunistic pathogen, was mainly responsible for mandarin fish haemorrhagic septicaemia. In this study, histopathological examination and immune-related gene expression statuses of *A. hydrophila* infected mandarin fish were investigated and the examination revealed inflammation, vacuolization, and extensive necrosis in the diseased fish's gills, liver, spleen, head, and kidney. After *A. hydrophila* infection, mRNA regulation for six immune-related genes was measured using quantitative real-time PCR in mandarin fish, and the transcriptional analysis of these immune-related genes revealed that the expression levels of MHC II, TCR, TNF, CC chemokine 3, interleukin 8 (IL-8) and Hepcidin were greatly up-regulated in the spleen, head, and kidney of mandarin fish after infection (Chen *et al*., 2018).

The effects of dietary curcumin on grass carp growth performance, general immunity, antioxidant capacity, and associated gene expression of NF-B and Nrf2 signalling pathways were examined in the previous study. *A. hydrophila* was used to infect fish, and the mortality rates were tracked for 7 days. Administering *A. hydrophila* to grass carp, with dietary curcumin has helped to increase the reduced expression of glutathione (GSH) content and also increased the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and

glutathione reductase (GR), decreased reactive oxygen species (ROS), and malondialdehyde (MDA). Supplementing the food with adequate amounts of curcumin may help juvenile grass carp grow, lower FCR, improve disease resistance, innate immunity, and antioxidant capacity in fish, and attenuate inflammatory response (Ming *et al*., 2020).

Temperature as a stress condition in fishes was studied by many. Some of the works for instance are like in the study in which was mainly conducted to understand how fish experience oxidative stress with temperature. Malondialdehyde (MDA), a lipid peroxidation degradation product, and catalase activity were monitored during the prolonged experiment in white muscle. The pattern for MDA and also catalase activity was the same. With time, MDA along with catalase activity both increased at 18° C. MDA levels and catalase activity were at a lower level at 24 °C than they were at 18 °C, as was the rate of concentration increase over time. MDA and catalase increased at 28 °C. The temperature was not a direct predictor of the oxidative stress response. It decreased at the ideal temperature (24 °C) and increased beyond the upper and lower ideal temperature limits for this species (Vinagre *et al*., 2012).

In agreement with the current study, another work was conducted to identify *Onychostoma macrolepis's* antioxidant defences in response to heat stress and to understand how the antioxidant system helped *O. macrolepis* adapt to it. Experimental fish were challenged at 30 °C for different periods respectively, after becoming acclimated at 24 °C. The experimental fish underwent various levels of stress, and the activity of SOD and CAT, as well as changes in the expression pattern of Cu/Zn superoxide dismutase (Cu/Zn-SOD) and also catalase (CAT), were assessed. The mRNA expression of Cu/Zn-SOD and CAT considerably increased in different tissues in the heart, liver, spleen, kidney, intestine, and muscle of *O. macrolepis*, showing a sensitive response of Cu/Zn-SOD and CAT to heat exposure, when the water temperature was elevated from 24 °C to 30 °C. In different tissues, Cu/Zn-SOD and CAT mRNA expression varied, indicating that the tissues' susceptibility to thermal stress differed. The antioxidant defences of *O. macrolepis* are enhanced as a result of the antioxidant system's fast reaction to short-term heat stress at 30 °C in the form of both Cu/Zn-SOD mRNA expression and also the activity of CAT and SOD (Yu *et al*., 2017).

The abiotic component temperature has an impact on a variety of biological and physiological processes in fish. Temperature stress is known to increase the production of

reactive oxygen species (ROS), which causes oxidative stress. Fish have developed a system of antioxidant enzymes to reduce the damaging effects of ROS. Fish are protected from oxidative stress by the major enzymes in the glutathione peroxidase (GPx) family. The findings showed that lower temperatures caused GPx1 gene expression to be down-regulated. The expression of the GPx1 gene varied little between fish exposed to high temperatures and control fish, though. The first information on GPx gene expression in *Tor tambroides* under heat stress is provided by this work. In this study; they discovered the full-length GPx1 cDNA and examined the patterns of GPx1 mRNA expression in response to temperature variations in the liver and muscle of *T. tambroides*. When compared to the control temperature (28°C), the levels of Tor-GPx gene expression indicated a downward trend under low temperature (11°C) and no changes under hot temperature (38°C). This study implies that cold temperature stress, which decreased metabolic rate, affected Tor-GPx1 gene expression (Do *et al*., 2019).

Wild albino northern snakehead, *Channa argus* was studied under temperature changes and bacterial infection, and the heat shock protein 70 (CaHSP70) protein was cloned from a 2,213 bp cDNA in this study. CaHSP70 has an open reading frame (ORF) that can encode 639 amino acids, and the corresponding polypeptides are 70.50 kDa. In the control groups, the CaHSP70, CaHSC70, and CaHSPA9 genes' mRNA expression levels varied depending on the tissue. In the kidney, liver, spleen, and brain tissues after the heat shock at 37 °C, the mRNA expression levels of CaHSP70 were extremely dramatically increased, although less of CaHSC70 and CaHSPA9 exhibited a comparative induction in these tissues. After *Edwardsiella tarda* infection, there was a general trend of first up regulation and subsequently a decline in the expression levels of CaHSP70, CaHSC70, and CaHSPA9.This study showed that CaHSP70, CaHSC70, and CaHSPA9 mRNA expression levels were significantly influenced by temperature and that these three genes' mRNA expression levels were susceptible to pathogen infection, particularly CaHSP70 in the albino *C. argus* (Zhou *et al*., 2020).

There are many stressful conditions other than bacterial stress are increasing nowadays. They include hyperoxic condition, increased lead concentration, indoxacarb presence, micro plastics influences were studied. And for instance, in the previous study in which three antioxidant genes like Cu/Zn superoxide dismutase (SOD), catalase (CAT), and also phospholipid hydroperoxide glutathione peroxidase (GSH-Px)— mRNA levels were assessed

in Atlantic salmon after the exposure to hyperoxia. The levels of oxidized glutathione (GSSG), total glutathione (GSH), and the consequent oxidative stress index (OSI) in blood were assessed as classic oxidative stress markers. After exposure to hyperoxia at the two sampling times, no significant mean normalized expression (MNE) changes of SOD, CAT, or GSH-Px were discovered in the liver. SOD, CAT, and GSH-Px mRNA expression in the liver had poor correlations with hyperoxic exposure regimes (Olsvik *et al*., 2005).

In the previous study in which rainbow trout macrophages were used as an in vitro model to examine the effects of copper and bacterial lipopolysaccharide (LPS), separately and together, on the transcription of target genes important for the immune system, respiratory burst activity, and cell death. Both wild and farmed fish may be simultaneously exposed to multiple stressors in their habitats, such as chemical toxins (like heavy metals) and infections (like bacteria). Results of the work showed that, except for the cell-death HMGB gene, copper induces all of the examined genes in HKM in a dose-dependent manner. Cytokines were often expressed more when LPS was present. LPS exposure also makes acute phase protein genes (SAA) and oxidative stress genes responsive. Synergistic effects in cytokine gene response were observed when both stimuli were delivered simultaneously at the concentrations of 1 and 10 uM Cu, but not in oxidative stress or cell death genes (Teles *et al*., 2011).

In the previous work in which response of the mudskipper was tested for eight days at four different lead concentrations. Fish exposed to 2 mg/L Pb exhibited the lowest liver and gill glutathione (GSH) and glutathione peroxidase (GPX) activity. Compared to fish exposed to 0 and 0.5 mg/L Pb, fish subjected to 1 and 2 mg/L Pb showed reduced liver superoxide dismutase (SOD) levels. Malondialdehyde (MDA) levels were highest in the liver and gills of the 2 mg/L Pb group. Heat shock proteins 70 (HSP-70), glutathione s-transferase (GST), and HSP-90 expression were all down regulated in the gill and liver of the 0 mg/L Pb group. Catalase (CAT), GPX, and GSH activities in gill were at their lowest point over the eight days on day eight. On day 8, the liver's CAT activity was at its lowest and its MDA concentration was at its highest. On day 2, the levels of SOD and HSP-90 expression in the gill were at their greatest. But from days 6 to 8, the liver's HSP-70 expression levels peaked. This study showed that antioxidant enzymes do not completely prevent Pb-induced ROS production. mRNA expression has an impact on the activity of functional enzymes (Jing *et al*., 2017).

The effects on immune, antioxidant, and expression of a stress gene in common carp after chronic indoxacarb exposure were examined in this study. Indoxacarb increases the expression of inflammatory cytokine genes (IL-1, IL-8, IL-10, TNF-, and IFN-) at low concentrations while inhibiting inflammatory cytokine expression at elevated concentrations. The analysis of SOD and CAT gene expression, that is antioxidant genes in various organs shows that low doses of indoxacarb enhanced their expression to deal with the primary oxidative situation. But studies show that higher indoxacarb concentrations reduced oxidative gene expression. Exposure to indoxacarb has an effect on the health of common carp at the transcriptional level. Gene expression variations suggest that exposure to indoxacarb interfered with the inflammatory response produced oxidative stress, and caused tissue damage (Ghelichpour *et al*., 2019).

The size impact of micro-plastic polystyrene on certain physiological injuries in the goldfish *Carassius auratus* was investigated. Two distinct sizes of polystyrene were challenged in fish at various ecologically appropriate quantities. Observations made with a fluorescent microscope supported the finding that tissue samples over time accumulated more polystyrene. Fish exposed to polystyrene had an increase in SOD and CAT activity as well as a notable variation in the expression of genes involved in the antioxidant system (CAT, SOD, and HSP70). The outcomes supported the harmful effects of polystyrene and micro plastic on goldfish (Abarghouei *et al*., 2021).

Different types of bacterial diseases are increasing nowadays. Many works were conducted on different bacterial attack on fishes. For instance, the work in which Peatmen *et al*, (2020) assessed the APR in channel catfish liver following infection with *Edwardsiella ictaluri*, a bacterial pathogen that results in intestinal septicaemia in catfish, using a novel high-density in situ oligonucleotide microarray. Using a standard 2-fold change in gene expression cut-off and a 10% false-discovery rate, the microarray data analysis revealed that catfish have a well-developed APR, with particularly strong up regulation (>50-fold) of genes associated with iron homeostasis (i.e. intelectin, hemopexin, haptoglobin, ferritin, and transferrin). Transport proteins, complement components, proteinase inhibitors, and coagulation factors were among the other canonical APP genes with higher than 2-fold up regulation. The membrane assault complex components and complement inhibitors were among the complement cascade

components that were upregulated. Following infection, the expression of many chemokine and pathogen recognition receptors (PRRs) was also altered (Peatman *et al*., 2007).

In the study, three zebra fish PGRPs were discovered, cloned, and evidenced to be significantly expressed in developing embryos, adult tissues exposed to the environment, and eggs. Uniquely, peptidoglycan-lytic amidase activity and broad-spectrum bactericidal activity are both present in zebrafish PGRPs. Furthermore, they showed that one of these PGRPs is crucial for protection and survival against bacterial infections in the growing zebrafish embryo. These findings show that PGRP protein is necessary for vertebrate life as well as innate immunity's protection against infections in fish embryos. Results show that innate immunity is crucial for fish infection defence, and they also pinpoint the part of their innate immune system that has this effect. The fact that PGLYRPs are expressed in so many different tissues shows that this family of proteins plays a significant part in protecting adult zebra fish from bacterial infections (Li 2007).

Hemibarbusmylodon's manganese superoxide dismutase (Mn-SOD) genomic structure was analysed. They noticed that Mn-SOD expression altered in response to a variety of stimuli, including as the lipopolysaccharide (LPS) challenge, bacteria, and heavy metals. LPS injection, *Edwardsiella tarda* challenge, and heavy metal exposure all dramatically altered Mn-SOD expression in the liver and kidney. According to this study, Mn-SOD is critical for the host's defence against oxidative stress brought on by infection-mediated inflammation and/or toxicant-related stress in this species (Cho *et al*., 2009).

To cause edwardsiellosis, healthy Pangasianodon were sub-lethally infected intramuscularly with *E. tarda*. The mortality was noted 48 hours after the injection. Over a 20-day observation period, the average mortality rate was 42% in fish exposed to *E. tarda* cells, compared to 6.6% in the placebo control group (catfish received 0.1 mL of 0.85% sterile physiological saline), and 0% in the negative control group. After infection, infected fish showed significant up-regulation of the IL-1 and C3 gene expression in their liver, kidney, spleen, and blood, however, the magnitude of the up-regulation varied depending on the organ and dpi. At 1 dpi, the kidney, spleen, and blood showed significant up-regulation of transferrin gene expression while the liver showed no significant change (Hoque *et al*., 2020).

The effects of dietary hawthorn extract (HTE) supplementation on *Trachinotus ovatus* growth performance, immunological responses, hepatic antioxidant capacity, growth- and immune-related, and heat-shock protein gene expression. The transcription rates of growth-related genes (IGF-I and IGF-II) were significantly up-regulated in fish given HTE supplements, in contrast to HSP70 and HSP90 mRNA levels (P 0.05).Antioxidant enzyme genes (CAT, GPx, MnSOD, and Keap1) as well as cytokines (IL-10, TGF-1) and TOR cytokine production were down regulated. According to the findings, golden pompano fed a diet supplemented with 0.50 g kg1 HTE may considerably improve growth performance and the expression of growth-related genes, boost immunity, and enhance hepatic antioxidative capacities and resistance to *Vibrio harveyi* infection (Tan *et al*., 2017).

There are lots of works in which effectiveness of various naturally available treatment measures for different stress conditions was studied. For instance, the study looked at Ficuscarica polysaccharide's impact. The impact of FCP considerably (P 0.05) up regulated the expression of IL-1 and TNF, according to the data. After the study, fish-fed FCP had considerably (P 0.05) reduced levels of HSP70 gene expression. FCP-fed grass carp had noticeably greater resistance to *F. columnare* (60% survival) than the control group (30% survival). Results show that FCP can increase the expression of immune-related genes, boost the immune system, and thus improve grass carp disease resistance (Yang *et al*., 2015).

The previous study assessed the results of dietary –glucan. The survival rates of rainbow trout *(Oncorhynchus mykiss*) infected with *Aeromonas salmonicida* as well as the mechanisms governing stress and immune-related parameters were examined. One of the most well-known immunostimulants, -glucan, has been shown to boost the innate response with no impact on animal development. After *A. salmonicida* infection, survival rates in -glucan groups dramatically improved in comparison to the control group (LiqinJi *et al*., 2017).

To investigate *Macrobrachium rosenbergii's* response to the leaf of *Moringa oleifera* extract when exposed to a lot of ammonia was analysed. Four treatment groups received a basic diet containing *oleifera* leaves extract and enrofloxacin, while a control group received a basal diet alone. Freshwater prawns were then exposed to extreme ammonia stress and infection with *Vibro anguillarum.* According to the findings, when compared to the control group, results shows *M. oleifera* leaf extract, when added at a rate of 0.5%, improves growth, even has favorable impacts on physiological and immunological function, and protects freshwater

prawns from severe ammonia stress. Antioxidant activities of genes like superoxide dismutase (SOD), and catalase (CAT) have enhanced at this particular concentration (Kaleo *et al*., 2019).

In the previous research, they looked at how probiotic bacteria affected the immunological response of pathogen-infected primo culture trout intestinal cells. In their research, scientists examined the impact of pre-treating intestinal cells with an indigenous strain of *Lactobacillus plantarum* after exposure to the two most dangerous salmonid infections—*Aeromonas salmonicida* and *Yersinia ruckeri*—on the pathogens' ability to cause disease. By the reduced expression of pro-inflammatory cytokines and expression levels of anti-inflammatory cytokines, the investigated probiotic strain reduced inflammation following *A. salmonicida* infection. In contrast, the probiotic strain promoted immunity by up-regulating the expression of pro-inflammatory cytokines and suppressing the activity of anti-inflammatory cytokines after a challenge with *Y. ruckeri*, which results in immunosuppression (Maruscakova *et al.*, 2021).

MATERIALS AND METHODS

SAMPLE COLLECTION

Molly fishes (*P. spenops*) were collected and treated with bacterial stress along with the control. Bacteria (*A. hydrophila)* was dissolved in 1ml of autoclaved water and inoculated to the bowl containing 500ml of water at 27℃ and 8.5 Ph. After 45 hours sample tissues were isolated and further experiments were conducted.

GLASS WARES AND OTHER EQUIPMENT

Glass bowls, fish feed, vinyl gloves, Collection tubes, tips, pipettes, Plastic wares, parafilm, PCR tubes petri plates, motor, and pestle,

RNA EXTRACTION

Precautions to be taken while handling RNA:

1. To avoid RNAse contamination from the skin surface or dusty laboratory equipment, every time worn latex or vinyl gloves when handling chemicals and RNA samples.

2. To avoid any contamination from RNAses through shared equipment, used only sterile, plastic disposable ware and micropipettes designated for RNA work.

3. Before usage, non-disposable plastic items were treated to remove RNase.

4. Glassware used for RNA research were well cleaned with detergent, autoclaved or heated for 15 minutes at 100°C

Procedure:

1. Sample preparation:

   Ground the fish tissue sample in a motor and pestle with liquid nitrogen and 1ml of Trizol

   Transferred the mixture to a 2.0ml collection tube.

2. Phase separation:

   a.The homogenized sample was incubated for a period of five minutes at room temperature (15–25 C) to allow the nucleoprotein complexes to completely dissociate.

   b. 200µl of chloroform per ml of Trizol reagent is added. Cover the sample tightly, shake vigorously for 15 seconds, and allowed the tube to stand for 10 minutes at room temperature (15-25˚C).

   c. Centrifuged the resulting mixture at 13,000 rpm for 15 minutes at 4˚C. Following centrifugation, the mixture separates into a lower organic phase (containing protein), an interphase (containing cell debris and DNA), and an upper aqueous phase containing RNA.

3. RNA precipitation:

   a. Transferred the aqueous phase containing RNA to a fresh tube and add 500µl of isopropyl alcohol. Allow the sample to stand for 10 minutes at room temperature (15-25˚C).

   b. Centrifuged at 12,000 rpm for 10 minutes at 4˚C. Before centrifugation, the RNA precipitate, which is usually undetectable, forms gel-like pellets on the tube's side and bottom.

4. RNA wash:

   Removed the supernatant and washed the RNA pellet by adding 1 ml of 75% ethanol. Pipetted gently to resuspend the pellet and centrifuged at 10,000 rpm for 5 minutes at 4˚C.

5. RNA elution:

   Briefly dried the RNA for 10 minutes by air-drying. Resuspended the pellet with 50µl of RNase-free water.

   Storage of the elution with purified RNA: Elution containing purified RNA should be kept at a very lower temperature (-80 °C) because it includes just pure RNA. Avoid repeatedly freezing and thawing the sample to prevent RNA from becoming denaturized.

cDNA SYNTHESIS

PrimeScript™ RT reagent Kit (Perfect Real Time) :

Reverse transcription for real-time RT-PCR is carried out using the PrimeScript RT reagent Kit. This makes use of the excellently extendable PrimeScript Reverse Transcriptase. The kit enables extremely quick and effective cDNA template generation for real-time PCR. This kit's experimental steps are straightforward and appropriate for high-output analysis. This kit can be used in association with a real-time PCR reagent based on an intercalator. Here it is used or TB GreenPremix Ex Taq (Tli RNaseH Plus) Mix, for 2 steps of real-time RT-PCR.

Reverse transcription for real-time RT-PCR is carried out using the PrimeScript RT reagent Kit. This makes use of the excellently extendable PrimeScript Reverse Transcriptase.

Protocol:

1. Prepared the following reaction mixture on ice. Created slightly more master mix than necessary to account for pipetting errors. After dispensing aliquots of this mixture into the microtubes, added the RNA sample.

Control (T2C)

Table1: Contents of reaction mixture for control

Reagent	Volume	Final conc.
5X PrimeScript Buffer (for Real Time)	2µl	1X
PrimeScript RT Enzyme Mix I	0.5 µl	
Random 6 mers (100 µM)*1	0.5 µl	50 pmol
total RNA	5.5 µl	
RNase Free dH2O	1.5 µl	
Total		10 µl

Test (T2T)

Table2: Contents of reaction mixture for test

Reagent	Volume	Final conc.
5X PrimeScript Buffer (for Real Time)	2µl	1X
PrimeScript RT Enzyme Mix I	0.5 µl	
Random 6 mers (100 µM)*1	0.5 µl	50 pmol
total RNA	0.5 µl	
RNase Free dH2O	6.5 µl	
Total		10 µl

2. Incubated the reaction mixture under the following condition.

- 37℃ 15 min (Reverse transcription)
- 85℃ 5 sec (Heat treatment inactivates reverse transcriptase)
- 4℃ at hold

REAL-TIME PCR

Real-time PCR is a technique for continuously monitoring the progress of a PCR reaction. Simultaneously, a small amount of PCR product (DNA, cDNA, or RNA) can be quantified. A reporter molecule emits fluorescence, which is detected by real-time polymerase chain reaction (PCR) as the reaction develops. This happens because the PCR product accumulates with each cycle of amplification. These fluorescent reporter molecules contain dyes that bind to double-stranded DNA (for example, SYBR® Green). This allows one to start with small amounts of nucleic acid and accurately quantify the end product. The development of the PCR is observed using a fluorescent reporter molecule. The reporter molecule's emitted fluorescence multiplies as the PCR product builds up after each round of amplification.

Table 3: List of primer combinations used for the Real time PCR

GENE	FORWARD PRIMER SEQUENCE	REVERSE PIMER SEQUENCE
CAT	TCCTGAATGAGGAGGAGCGA	ATCTTAGATGAGGCGGTGATG
GST	TAATGGGAGAGGGAAGATGG	GAGCTTCATGCCATCCATTT
SOD	GGTGCCCTGGAGCCCTA	GCAACCTGTGTTGTCACGTC
HSP 70	GGGAGAGGGTTGGGCTAGAG	TTGCCTCCTGCCCAATCA
GPX	CGCCGAAGGTCTCGTTATTT	TCCCTGGACGGACATACTT
GAPDH	CGTAGCTGACTTTTCTGGATTACG	GCAGCTATGACATCATGAAATGAA

Real-Time PCR setting

Reaction Volume - 6ul

2 X Real-Time PCR smart mixes- 3ul

Forward primer + Reverse primer-0.5ul

Template cDNA - 1ul

ddNFW - 1.5ul

The contents are mixed thoroughly and spinned downed using vortex mixture.

PCR Program

Table 4: Real time PCR amplification program

Steps	Temperature	Time	**Cycle**
Polymerase activation	95 °C	10 minutes	
Denaturation	95 °C	15 sec	40 cycles
Annealing/Extension	72 °C	20sec	

Real-Time PCR – Fold of Induction:

The response, measured as the fold of induction. That is fold change is a measue describing how much quantity changes between an original and subsequent measurement.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control - sample)}}$$

Ratio = target gene (ct target of control – ct target of treatment)

Reference gene (ct reference of control – ct reference of treatment)

Fig 1: Experimental set up showing fish inoculated with a

Fig 2: Fish killed for tissue extraction

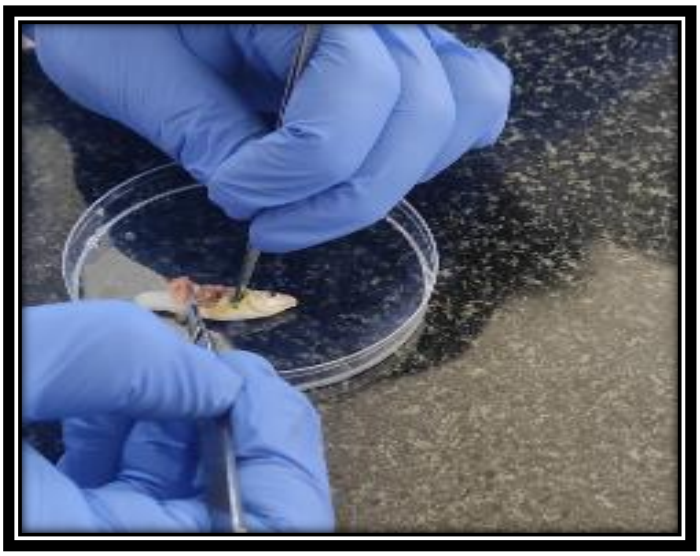

Fig 3: Tissue extraction for RNA

Fig 4: grounding tissue sample using motor and pestle

RESULTS

CLINICAL MANIFESTATIONS

They showed vigorous movement on the 2nd day of *A. hydrophila* infection. Later they seemed to be normal may be because of the inherent defensive mechanism.

1. RNA concentration.

   RNA from the samples and control were isolated using the Trizol method (Takara, Japan). Isolated RNA of concentration 2289.0 (ng/ul) from the test sample was obtained.

Table 5: Nanodrop concentrations obtained from each sample

Sample	Concentration(ng/ul)
Control(T2C)	1285.0
Test(T2T)	2289.0

GENE 1

1. Real-Time / qPCR Analysis.

CQ values obtained, mean CQ, PCR efficiency, and fold of induction of the gene are given below. The melt curve and melt peak figures are attached.

Catalase (CAT) is a most common enzyme that is usually found in many organisms. It has an anti-oxidant activity and can be used as a biomarker. Catalase is usually involved in decomposing hydrogen peroxide into water and oxygen. It is clear from the values obtained; that the fold of induction of the test (1174.470561) is higher than the control (1) (Table 2) in reference to the housekeeping gene, and it represents the upregulation of the CAT gene in fish infected with Aeromonas hydrophila.
Thus the immune system stimulated and protects the cell from oxidative stress due to bacterial infection.

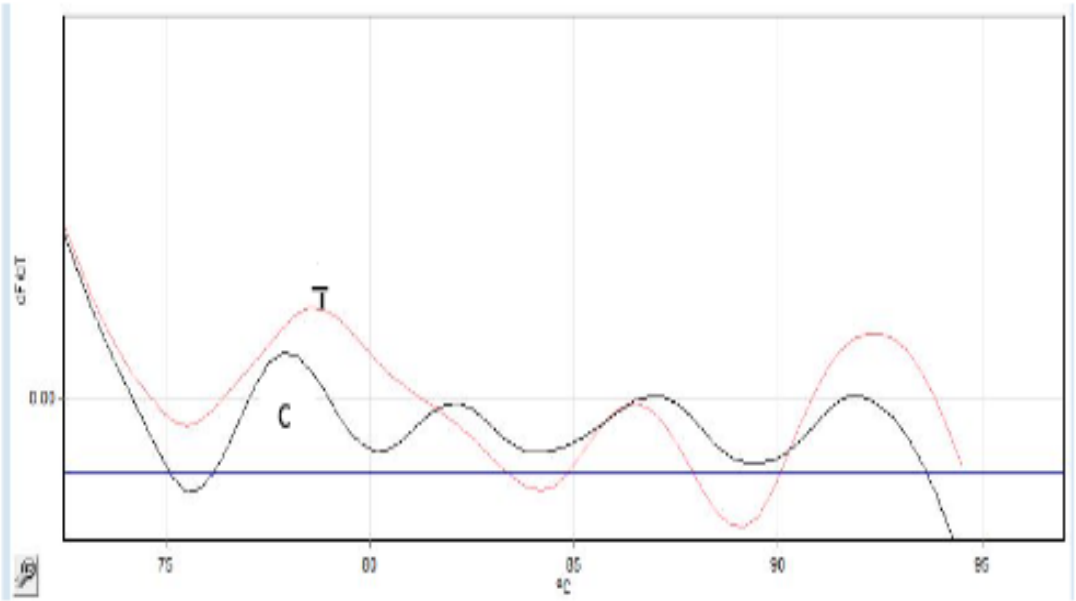

Fig 5: Melt curve analysis of CAT gene

Table 6: Ct of target (CAT), reference gene (GAPDH), and fold induction

	Target gene	Reference gene	
PCR efficiency	1.4680	1.4260	
Ct values	Ct values	Ct reference	Fold induction
T2C	26.81	25.31	1
T2T	6.90	23.69	1174.470561

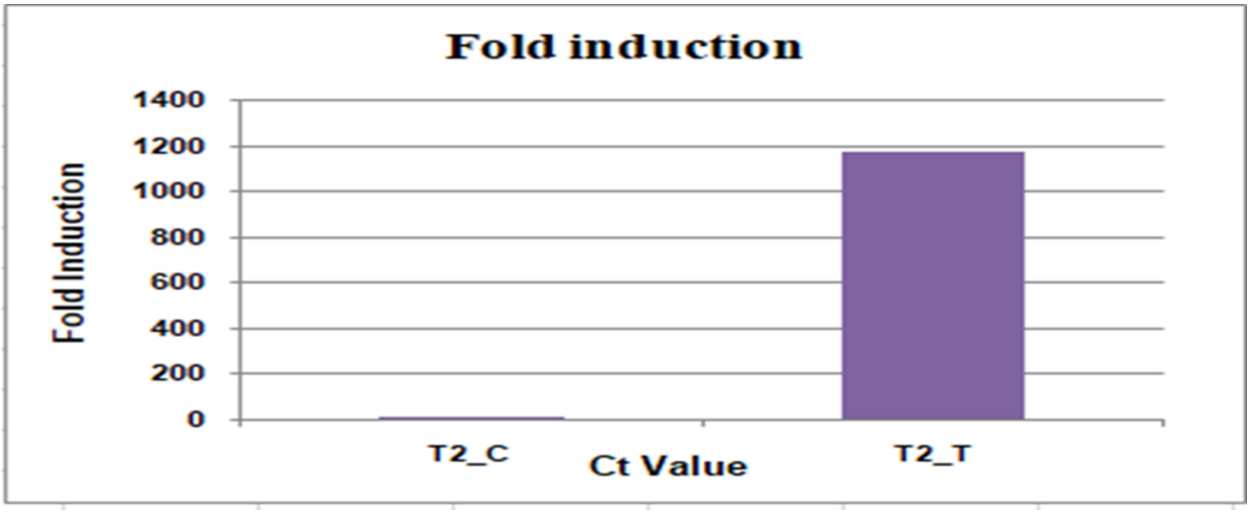

Fig 6: Fold induction of CAT gene

GENE 2

Ct values obtained, mean Ct, PCR efficiency, and fold of induction of each gene are given below. The melt curve and melt peak figures are given below.

Superoxide dismutase (SOD) is an antioxidant enzyme that is actively involved in the defensive mechanism of most organisms and hence it can be considered a biomarker. In the current study, it is observed from the values that the fold of induction of the test sample is lower (0.1051316561) than that of the control (1)(Table 3), in reference with the housekeeping gene. So it is understood that the bacterial infection can down-regulate the SOD gene which in turn increases the oxidative stress by producing more reactive oxygen species (ROS).

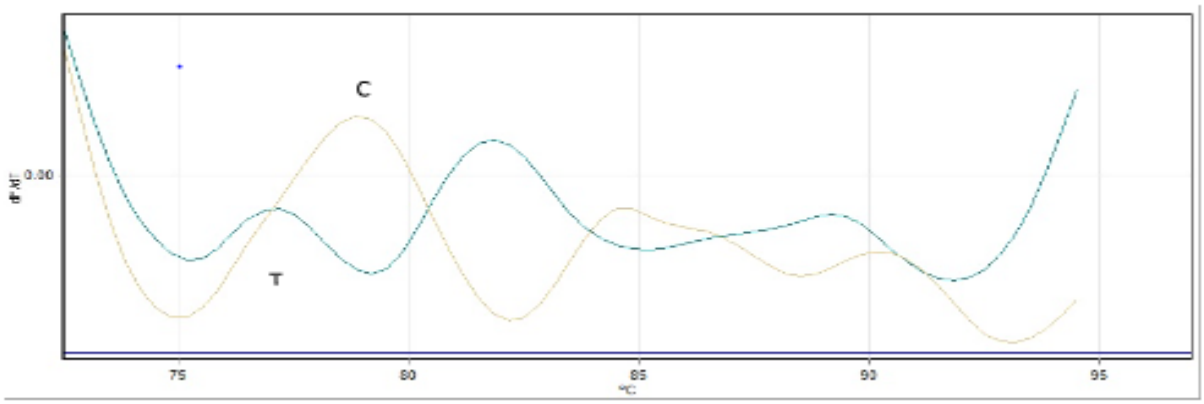

Fig 7: Melt curve analysis of SOD gene

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Table 7: Ct of target (SOD), reference gene (GAPDH), and fold induction

	Target gene	Reference gene	
PCR efficiency	1.4680	1.4260	
Ct values	Ct target	Ct reference	Fold induction
T2C	6.92	25.31	1
T2T	11.29	23.69	0.1051316561

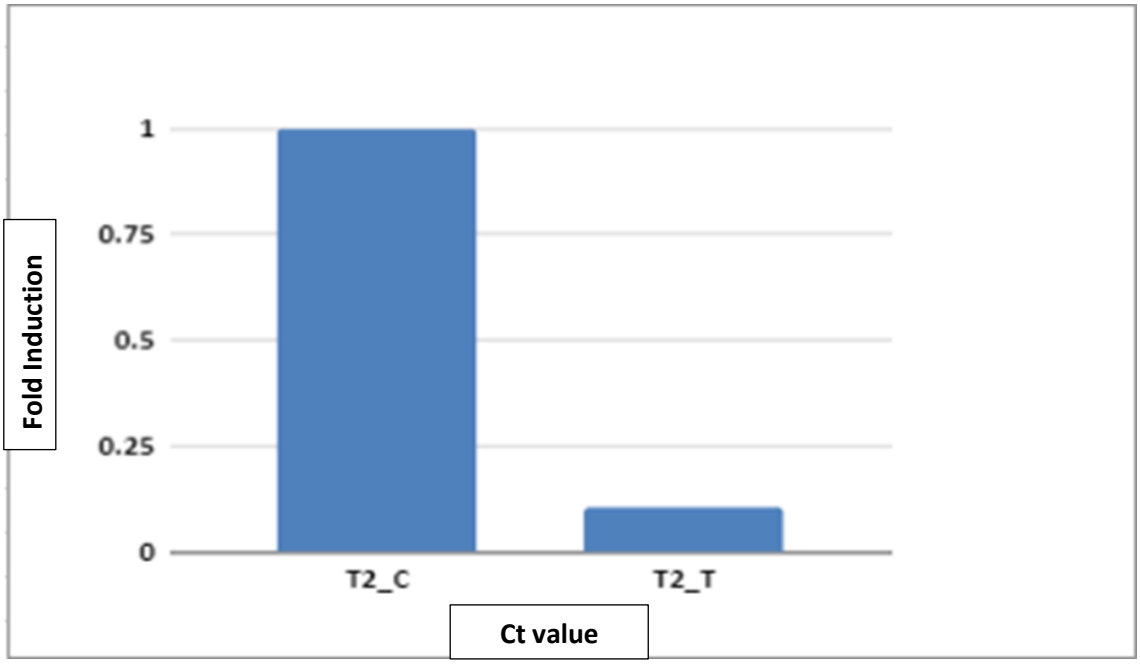

Fig 8: Fold induction of SOD gene

DISCUSSION

The present study was conducted on Molly fishes (*P. sphenops*) belonging to the family Poeciliidae, when infected with *A. hydrophila*, a pathogen that can cause infectious diseases and is a major cause of economic loss in commercial aquaculture. The survival ability of fish when inoculated with a trace amount of bacteria has given information regarding the regulation of different genes like CAT and SOD during the infection. The influence of any bacterial stress could lead to the overproduction of reactive oxygen species (ROS) which results in oxidative stress in fish (Ellis, 1998). An increase in the level of ROS May causes lipid peroxidation which likely damages cellular molecules (Sanchez *et al*., 2005). To minimize the negative impact of ROS, fishes use several defence mechanisms that are either enzymatic or non-enzymatic Zaleski *et al*., (2014). Catalase is an antioxidant enzyme that is part of enzymatic mechanisms involved in detoxification by breaking down the toxic reactive oxygen species (ROS).

Here in this study CAT gene shows a significant up regulation due to the bacterial stress, were as SOD genes are down regulated.

Bacterial stress could enhance gene regulation as a primary defence mechanism as observed in the works of Das *et al*., (2011). They studied the innate immune responses of *P. sarana* after infection with *A. hydrophila*. The liver of the survivors had a noticeable up-regulation of C3 and transferrin was identified. Another work done by Zhou *et al*., (2020) on Channa fish infected with *E. tarda* shows up regulation of CaHSC70 mRNA, it rose first, then fell, and finally reached its highest value after a certain period following infection. This phenomenon suggests an inherent defence mechanism. Similarly in the work by Chen *et al*., (2018) in which mandarin fish were challenged with *A. hydrophila* was conducted and the results revealed that the expression levels of MHC II, TCR, TNF, interleukin 8 (IL-8), CC chemokine 3, and Hepcidin were highly up-regulated in the spleen and head kidney of mandarin fish after infection. *E. ictaluri*, a bacterial pathogen that results in intestinal septicemia in catfish, when infected, data analysis revealed that the Catfish have a strong upregulation of genes associated with iron homeostasis (i.e. intelectin, hemopexin, haptoglobin, ferritin, and transferrin) Peatman *et al*., (2007). Pangasianodon were sub-lethally infected intramuscularly with *E. tarda* by Hoque *et al*., (2020). After infection, infected fish showed significant regulation of the IL-1 and C3 gene expression in their liver, kidney,

spleen, and blood; however, the magnitude of the up-regulation varied depending on the organ.

All these work in which different fish challenged with different bacterial pathogen showed significant up regulation in various genes. Similarly, the current study, showed a significant up regulation in Catalase (CAT) gene in *P. sphenops* when infected with *A. hydrophila*, which provides information regarding the inherent defence mechanism during bacterial stress.

As an impact of stress, some genes could get down regulated. For instance, the work by Das *et al*., (2011) infected *P. sarana* with *A. hydrophila*. Down-regulation of lysozyme G, interleukin 8, MnSOD, and B2M was observed in the liver after the infection. *T. tambroides* are stressed with temperature in the work done by Do *et al*., (2019). When compared to the control temperature (28°C), the levels of Tor-GPx gene expression indicated a downward trend under low temperature (11°C) and no changes under hot temperature (38°C). The response of the mudskipper was tested at four different lead concentrations by Jing *et al*., (2017). The expression of several genes like Heat shock proteins 70 (HSP-70), glutathione s-transferase (GST), and HSP-90 expression were all downregulated in the gill. The work showed that antioxidant enzymes do not completely prevent Pb-induced ROS production.

In the current study also superoxide dismutase (SOD) have been significantly downregulated and Glutathione peroxidase (GPX), Glutathione S Transferase (GST), and heat shock protein 70 (HSP70) got slightly down regulated. which may represent the harmful effect of bacterial infection.

There are several works were conducted on fish to study their gene regulation due to different stress rather than bacterial stress. The effects on immune, antioxidant, and expression of a stress gene in common carp after chronic indoxacarb exposure were examined in the study by (Ghelichpour *et al*., 2019). Indoxacarb increases the expression of inflammatory cytokine genes (IL-1, IL-8, IL-10, TNF-, and IFN-) at low concentrations while inhibiting inflammatory cytokine expression at elevated concentrations. Antioxidant genes like SOD and CAT in various organs show that low doses of indoxacarb enhanced their expression to deal with the primary oxidative situation. But studies show that higher indoxacarb concentrations reduced oxidative gene expression. Similarly, in our current study in which *P. sphenophs* infected with a trace amount of *A. hydrophila* showed up regulation in the catalase (CAT) gene activity. But as mentioned in the previous study there may be a chance for the

CAT gene to get down regulated as the concentration of bacteria increases. As reported in the study by Chen *et al*., (2018), *A. hydrophila*, an opportunistic pathogen, was mainly responsible for mandarin fish haemorrhagic septicaemia. Here in our study, there are no severe symptoms noted may be because the observation period was very less even though some vigorous movements were shown by the fish on the second day of infection. If the time and concentration of the bacterial infection increased there may be a chance of showing the clinical manifestations.

In the study by Olsvik *et al*., (2005) in which Atlantic salmon exposed to hyperoxia, the mRNA expression of three different antioxidant genes like Cu/Zn superoxide dismutase (SOD), catalase (CAT), and also phospholipid hydroperoxide glutathione peroxidase (GSH-Px), were measured in their study. And in the other study by Refaey and Li, (2018) was to seek to determine how to transport stress affected the hepatic heat shock proteins (HSPs) of channel catfish. The findings showed that the mRNA expression of both HSP70 and HSP90 in the liver was significantly greater in the transported fish. The other study conducted on Zebrafish by Sarkar *et al*., (2014) which was used as a model to examine how oxidative stress-related enzyme activity and antioxidant gene expression in the brain due to toxic arsenic trioxide Catalase (Cat), copper/zinc superoxide dismutase (Cu/Zn Sod), manganese superoxide dismutase (Mn-Sod), and cytochrome c oxidase 1 (Cox1) all had up regulated mRNA expression. In black porgy exposed to heat and hypo-osmotic stressors studied by An *et al*., (2010) and the activity of three antioxidant enzymes—Cu/Zn-superoxide dismutase (Cu/Zn-SOD), catalase (CAT), and glutathione peroxidase (GPX)—were measured to detect oxidative stress. After exposure, antioxidant enzyme expression and activity dramatically increased. In all these works different antioxidant enzyme expressions have been regulated due to different types of stress conditions in fish. Levels of expression of different genes in various fishes has changed according to the type of stress, catalase (CAT) has shown significant influence in the inherent defence mechanism in most of the stress condition and also in the current, so it helps understand the importance catalase gene.

The effects of dietary curcumin on grass carp growth performance, general immunity, antioxidant capacity, and associated gene expression of NF-B and Nrf2 signalling pathways were examined in by study by (Ming *et al*., 2020). *A. hydrophila* was used to infect fish, and the mortality rates were tracked. Administering *A. hydrophila* to grass carp, with dietary

curcumin helped to increase the reduced glutathione (GSH) content and also the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) thus decreased reactive oxygen species (ROS). They concluded that dietary curcumin can improve immunity by up regulating the down regulated genes. To investigate *Macrobrachium rosenbergii's* response to the leaf of *Moringa oleifera* extract when exposed to ammonia was analysed by Kaleo *et al*., (2019) and they identified when compared to the control group, results showed *M. oleifera* leaf extract, when added at a rate of 0.5%, improves growth, even has favourable impacts on physiological and immunological function, and protects freshwater prawns from severe ammonia stress.

Thus in our current study all the data, that is the catalase gene up regulation and SOD gene down-regulation provides baseline information for further studies to identify what therapeutic measures can be useful for increasing the gene expression and thus enhancing the immune system. For example to identify if any changes occurred for the down regulated gene after providing optimal dietary curcumin to molly fish, or any other naturally available measures can be tested and finally, an economically viable solution can be identified to prevent the economic loss due to the bacterial infection.

A. hydrophila, especially because the bacterium is a significant pathogen that affects all species of cultured fish in India and is responsible for several illness problems. From an Indian viewpoint, several experimental methods were used to identify a prospective vaccine candidate that might trigger immune defences in the host as well as the challenges involved in creating a good vaccine against these bacteria, as in the work by Nayak, (2020), it is very clear about the severity of the adverse effect of this pathogen. The current study provides baseline data about the gene expression of *A.hydrophila*. Information regarding gene expression from our work will be useful for future works on vaccine development.

CONCLUSION

Bacterial infection does always have a negative impact on the aquaculture industry. *A. hydrophila* is a gram-negative bacterium that is usually seen in freshwater bodies. The challenge can cause severe infection and also even fish death. Fish farming or aquaculture is a source of high protein and also it has great importance in the economy of the country. The pathogenic attack could lead to suppression of the immune system of cultured fishes and can cause severe infection and even death. Thus they have a negative influence on aquaculture in turn also affects the economy of the country.

In the current study, the impact of *A. hydrophila* in changing the magnitude of gene expression has been studied. We identified that the expression of catalase (CAT) gene has shown an upward regulation and the expression of superoxide dismutase (SOD) has shown a downward trend. The findings of the current study suggest that the bacterial infection has stimulated the inherent immune mechanism as well as it also affected the expression of some genes like SOD in a negative manner. This is a baseline work, which can be used for further studies to find a solution to this problem. By understanding the basic change in the antioxidant enzymes, it is possible to identify whether they are more up regulated like catalase (CAT) gene or down regulated like superoxide dismutase (SOD) in the current study. By up regulation of the catalase (CAT), here the enzyme tries to scavenge, neutralize, and/or detoxify ROS, thus organisms create defence mechanisms to defend themselves from "oxidative stress". Catalase is an antioxidant enzyme that is part of the enzymatic mechanisms involved in detoxification by breaking down toxic reactive oxygen species (ROS). The down regulation of the SOD and all other genes may be due to the negative impact of bacterial stress, which causes an increase in the level of ROS and could lead to lipid peroxidation, which likely damages cellular molecules.

The current study will contribute to understand the effectiveness of any treatment measures by analysing gene expression. The study will also be useful in understanding the effect of naturally available medicines, which is more economically suitable for the farmers to prevent economic loss due to this bacterial infection. Vaccine production against this pathogen (*A. hydrophila*) can utilize these results from the current study to evaluate the effectiveness of the vaccine which will be produced. Thus this work can be considered as a baseline work that contributes basic information for further future studies.

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