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Hematobiochemical Changes, Immunological Response and Immune Gene Expression in Nile Tilapia (*Oreochrom niloticus*) Experimentally Infected with *Shewanella putrefaciens*

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ABSTRACT

Background: In this work, hematobiochemical changes, immunological responses, and histopathological changes in Nile tilapia (*Oreochromis niloticus*) experimentally infected with *Shewanella putrefaciens* were studied.

Methods: *O. niloticus* was experimentally infected with *S. putrefaciens* (4SK/SRLFDA/19) at a concentration of 1.508×106CFU/ml per fish. Gross symptoms, namely hemorrhage on the body surface, skin discoloration, slow or nervous behavior, shallow necrotizing ulcers on the skin, fin erosions and abdominal distension were observed 5 to 6 days after infection. Fish were anesthetized and blood samples were collected at 0, 2, 4, 7, 10, 14, 20, and 27 days after the experimental infection to assess the hematobiochemical and immunological responses. For haematobiochemical responses, tests that included hemoglobin (Hb), leukocyte count (Lc), erythrocyte, hematocrite (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), cholesterol (CHO), glucose (GLU) and triglyceride were assessed.

Result: Values of hematocrit, hemoglobin, albumin, globulin, erythrocyte, MCHC, cholesterol and triglyceride were significantly (p<0.05) decreased. Lc, MCV, MCH, and glucose values were significantly (p<0.05) increased. Respiratory burst activity (RBA) and serum total protein values were significantly (p<0.05) decreased. Relative expression of immune gene toll like receptor 4 (TLR4), stress genes heat shock proteins 70, 90 (HSP70, HSP90), oxidative stress gene glutathione S-transferases (GST) and inflammatory gene interleukin 6 (IL6) was significantly higher in experimentally infected fishes compared to control.

Key words: Gene expression, Hematobiochemical parameters, Nile Tilapia, Respiratory burst activity, Serum total protein.

INTRODUCTION

Tilapia is considered as the second most farmed fish globally owing to its suitability for aquaculture and marketability (Wang and Lu, 2016). Tilapia is a candidate species for aquaculture in over more than 140 countries. Tilapia production in 2020 being 6,510,700 metric tonnes, which is predicted to increase to 7.3 million metric tonnes by 2030 (Singh, 2019). Nile tilapia (*Oreochromis niloticus*) contributed about 8.3% (4407.2 thousand tonnes) in 2020 in the total global aquaculture production (FAO, 2022). As in any other food production sector, diseases remain as the major challenge for aquaculture operations worldwide. It is reported that almost 50% production loss in tilapia farming occurs due to diseases (Assefa and Abunna, 2018). Currently there are more than 200 diseases identified in the cultured aquatic species, of which bacterial diseases are considered very important as they cause serious damage to the aquaculture operations and affect the livelihood of fish farmers. Shewanellosis is a bacterial disease of fish, which was first reported in 2002 in Poland (Kozinska and Pekala, 2004). The disease caused by *Shewanella putrefaciens* has emerged as an important bacterial pathogen in tilapia farming as it causes mortalities ranging from 30% to 80% and huge associated losses (Lu and Levin, 2010; Manal, 2017; Sood *et al.,* 2020). *S. putrefaciens* is a gram-negative bacterial pathogen known to cause systemic infections with histopathological changes in cultured fishes *viz.*, *O. niloticus, Dicentrarchus labrax, Misgurnus anguillicaudatus* and *Carassius auratusauratus* (Korun *et al.,* 2009; Qin *et al.,* 2014; Manal, 2017; Turgay *et al.,* 2014). The study was conducted to understand the hematobiochemical changes, immunological response and immune gene expressions caused in *O. niloticus* during an experimental infection with *S. putrefaciens.*

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MATERIALS AND METHODS

S. putrefaciens isolate

S. putrefaciens isolate (4SK/SRLFDA/19) obtained from the naturally infected *Tilapia* sp. which was maintained at

the repository of State Referral Lab for Aquatic Animal Health, Tamil Nadu Dr. J. Jayalalithaa Fisheries University (TNJFU) was used for experimental infection in this study.

An isolate (MW341434) was grown in a nutrient broth at 28°C for 18 h. The bacterial pellet was prepared by centrifugation (2000 g for 10 min) and suspended in phosphate-buffered saline (PBS). The stock culture with a concentration of 1×10^9 CFU/ml was prepared based on the optical density (OD) values observed at 600nm in a spectrophotometer (Shimadzu, Japan). Serial dilutions were prepared from the stock culture (10^8 to 10^3 CFU/ml) to assess its pathogenicity based on the LD50 assay (Reed and Muench, 1938).

Collection and maintenance of fish samples

Apparently healthy *O. niloticus* (weight 25±5 g) were acclimatized in the wet lab facilities of the State Referral Lab for Aquatic Animal Health, TNJFU–Madhavaram campus for 3 weeks prior to the experiment with sufficient aeration, optimum water quality parameters and feeding *ad libitum* with 30% crude protein feed twice a day.

Experimental challenge

Experimental challenge with *S. putrefaciens* isolate (MW341434) was carried out in the fish of the treatment group by injecting 1.508×10^6 CFU/ml, which was maintained in triplicates. Control fish received equal volumes of PBS. Observations on the moribund and dead fish were recorded in the experimental tanks daily for 4 weeks (Devi Kamilya and Abraham, 2012).

Hematobiochemical and immunological responses

Fish were anesthetized with MS-222 (20ppm) and blood samples (1 to 1.5 ml) were collected on days 0, 2, 4, 7, 10, 14, 20 and 27 post-infection to assess the hematological parameters *viz*, hemoglobin (Hb;g dl^{-1}), Lc ($\times10^3\mu L^{-1}$**)**, erythrocyte (million/$cumm^{-1}$), hematocrit%, mean corpuscular volume (MCV;fl), mean corpuscular hemoglobin (MCH;pg), mean corpuscular hemoglobin concentration (MCHC;g dl^{-1}) and biochemical parameters *viz.*, glucose (GLU;mg dl^{-1}), cholesterol (CHO;mg dl^{-1}), triglyceride (TG;mg dl^{-1}), respiratory burst activity (RBA), serum total protein (g dl^{-1}), albumin (g dl^{-1}) and globulin (g dl^{-1}). The erythrocyte (RBC) and leukocyte counts were analyzed by a Neubauer hematocytometer (Axiva Sichem Biotech, Delhi); Hemoglobin levels were determined by the cyanmethemoglobin method (Drabkin, 1946).

Hematocrit (Ht) and erythrocyte indices such as MCV, MCH and MCHC were calculated following the method of Nelson and Morris (1989) and Saravanan *et al.* (2011). The glucose concentration was assessed using a commercial kit (Sigma Diagnostics). Serum cholesterol and triglyceride concentrations were estimated following the method of Parekh and Jung (1970) and Rice (1970), respectively. RBA was estimated following the method of Stasiak and Baumann (1996). Serum total protein was determined based on the method of Reinhold (1953). Albumin and globulin levels were estimated by using a kit (Diatek, Kolkata).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the muscle and kidney tissues collected aseptically from fish using RNA iso-plus kit (Takara Bio Inc. Japan) following the manufacturer's instructions. The first-strand (cDNA) complementary DNA was created from 2 mg of total RNA by following the manufacturer's instructions for the first-strand cDNA synthesis reagent (Thermo Scientific, USA). To study the quantitative real-time (qRT-PCR), a β-actin transcript was used as an internal control. A C1000 Touch thermal cycler with a CFX96 Real-time PCR was used to conduct the qRT-PCR (Bio-Rad, USA). A specific set of primers was used to analyze gene expression (Table 1). 20 ng of cDNA template, 10 M of two primers (forward and reverse), 1x SYBR Green PCR Master Mix Kit (Takara Bio Inc. Japan), and 20 µl of nuclease-free water were used to perform RT-PCR. A relative expression level of the gene was displayed as $2^{-\Delta\Delta Ct}$ and calculated using the qRT-PCR cycle threshold (Ct) values (Livak and Schmittgen, 2001).

Statistical analysis

One-way analysis of variance and Tukey's test were conducted for multiple comparisons to study the variation among the groups on various days post-infection ($p<0.05$). The data were statistically analyzed by SPSS version 20.0, software (SPSS Inc.) for Windows.

RESULTS AND DISCUSSION

Behavioral changes *viz.*, swimming near the surface of water, lethargy, hemorrhages on the body, fin rot, and mortality were observed in *O. niloticus* experimentally challenged with *S. putrifaciens* 5 to 6 days post-infection. *Cyprinus carpio, Oncorhynchus mykiss* (Pezkala *et al.,* 2015), *O. niloticus* (Manal, 2017) and Seabass (Korun, 2009) infected with *S. putrefaciens* exhibited similar behavioral changes.

O. niloticus experimentally infected with *S. putrefaciens* showed significantly ($p<0.05$) lowered hematocrit and hemoglobin values compared to the controls from day 2 to day 4 post-challenge, respectively. Yu *et al.* (2010) observed a significant reduction of erythrocytes in peripheral blood, hemoglobin level, and hematocrit percentage, which are indices of anemia. *Aeromonas hydrophila* infection in spotted snakehead,*Channa punctatus* (Podeti and Benarjee, 2017), *Aeromonas* and *Streptococcus* sp. infections in cichlid fish also caused a reduction in Hb and RBC levels (Rehulka, 2002; McNulty *et al.* 2003).

Albumin and globulin levels were also lower than control. Decreased albumin level has been observed in *Labeo rohita* infected with columnaris disease (Tiwari, 2014). The leucocyte counts ($p<0.05$) were significantly higher in the infected *O. niloticus* than in the control fish from day 4 post-infection. Harikrishnan *et al.* (2010)

observed an increased leucocyte count in the gold fish infected with *A. hydrophila*.

A significant (p<0.05) increase in the MCV and MCH levels was observed in the infected *O. niloticus* than in control fish from day 7 post-infection. However, MCHC values (p<0.05) significantly reduced in the infected *O. niloticus* compared to uninfected control fish from day 10 post-infection (Table 2). Similar to our results, Ranzani-Paiva, *et al*. (2004) reported that there was a decrement in the RBC indicator MCHC in *O. aureus* challenged by *Corynebacterium* sp. In addition, Haniffa and Mydeen (2010) showed that catfish (*Silurus asotus*) revealed a decreased MCHC during *A.hydrophila* infection.

RBA and serum total protein levels showed significantly lowered values (p<0.05) in the infected *O. niloticus* than the control fish from day 2 post-infection (Table 3). A decreasing RBA trend was observed after bacterial infection in Rohu challenged with *E.tarda* (Mohanty and Sahoo, 2010). The reduction in TP has also been reported in various fish infected with pathogenic bacteria *viz.*, *Campylobacter cryaerophila, Arcobacter halophilus, Renibacterium* sp., *Flavobacterium* sp., *Streptococcus* sp., *Aeromonas* sp. and *Vibrio* sp. (Harbell *et al.,* 1979; Moyner, 1993; Aydin *et al.,* 2000; Rehulka 2002). Decreased serum total protein was observed in the present study following the observations made by Evenberg *et al.*

Table 1: Primers used for qPCR analysis of selected genes of Nile tilapia infected with *S.putrefaciens.*

Gene name	Primer sequence (5′-3′)	Reference
Glutathione S- Transferase	F: AAATGGATGGCATGAAGCTC	Costa *et al.,* 2012
	R: TCGTTCTTTGGGATCCTTTG	
Heat Shock Protein 90	F: ACGATGATGAGCAGTATGCC	Glencross *et al.,* 2016
	R: CAAACAGGGTGATGGGGTA	
Heat Shock Protein 70	F: CAAGGTGATTTCAGATGGAGG	Glencross *et al.,* 2016
	R: CTTCATCTTCACCAGGACCA	
Toll like Receptor 2	F: CCCACAATGGATTCACCAG	Korni *et al.,* 2021
	R: AAAGATCAAGACTCAAGGCACTG	
Toll like Receptor 4	F: TGTTCAAGATGCCACATCAG	Hsieh *et al.,* 2010
	R: TCCACAAGAACAAGCCTTTG	
Interleukin 6	F: ACAGAGGAGGCGGAGATG	Zou *et al.,* 2020
	R: GCAGTGCTTCGGGATAGAG	
b-actin	F: TACCACCGGTATCGTCATGGA	Rengmark *et al.,* 2007
	R: CCACGCTCTGTCAGGATCTTC	

Table 2: Hematological and biochemical parameters in Nile tilapia, injected with *S. putrefaciens.*

Infected group	Ht%	Hb (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})	Lc($\times 10^3$ μL^{-1})	Erythrocyte
Day 0	35.08±0.28f	11.4±0.15e	4.25±0.00g	4.09±0.00e	28.6±0.42a	4.81±0.05h
Day 2	34.06±0.57e	11.2±0.10e	4.22±0.00fg	4.09±0.00 e	29.07±0.47a	4.26±0.11g
Day 4	29.06±0.64d	9.1±0.15d	4.18±0.01ef	4.08±0.01e	32.7±0.05b	3.86±0.05f
Day 7	25.1±0.36c	8.06±0.15c	4.12±0.10e	3.88±0.90d	36.7±0.18c	2.86±0.05e
Day 10	11.13±0.05b	2.80±0.10b	4.01±0.00d	3.82±0.10d	39.1±0.17e	1.86±0.05d
Day 14	10.7±0.05ab	2.50±0.10ab	3.42±0.04c	3.21±0.08c	40.4±0.03f	1.46±0.05c
Day 20	10.06±0.05a	2.56±0.37 ab	3.17±0.05b	3.03±0.05b	41.3±0.09g	0.96±0.05b
Day 27	9.76±0.05a	2.26±0.15a	3.00±0.00a	2.05±0.03a	38.07±0.07d	0.73±0.05a

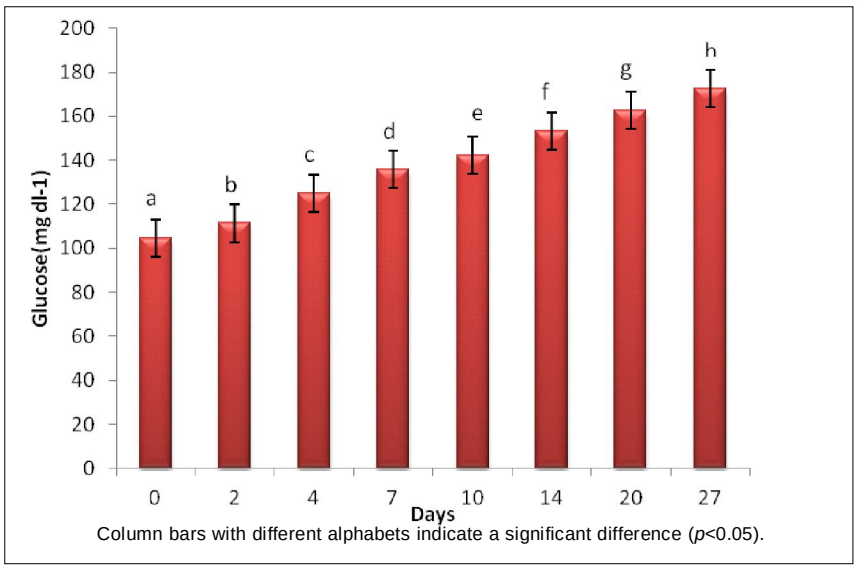
Table 3: Haematological and biochemical parameters in Nile tilapia, injected with *S.putrefaciens.*

Infected group	MCV (fl)	MCH (picograms)	MCHC (g dl^{-1})	RBA	Serum total protein (gdL^{-1})
Day 0	72.84±0.62b	23.73±0.26c	32.58±0.17b	1.46±0.00e	0.16±0.00g
Day 2	72.18±0.0b	25.80±0.53c	32.62±0.2b	0.15±0.00a	0.15±0.00fg
Day 4	75.17±1.62b	23.54±0.59c	31.32±0.97b	0.09±0.00a	0.14±0.00ef
Day 7	87.56±0.65c	11.69±0.06a	32.08±0.17b	0.08±0.10a	0.14±0.00de
Day 10	58.66±0.39a	15.47±0.23b	26.22±0.17a	0.12±0.02a	0.13±0.00cd
Day 14	73.2±3.3b	16.82±0.27b	23.29±1.04a	0.51±0.04b	0.12±0.00c
Day 20	104.3±5.8d	26.51±3.11c	25.48±3.6a	0.88±0.93c	0.10±0.00b
Day 27	133.6±9.7e	30.9±1.64d	23.2±1.59a	0.88±0.09d	0.07±0.01a

(1986) in *Cyprinus carpio* challenged with *Aeromonas salmonicida* and *Labeo rohita* infected with *Flexibacter columnaris* (Tiwari and Pandey, 2014). Glucose levels increased significantly (p<0.05) whereas cholesterol and triglyceride levels decreased in the infected fish (Fig 1-3). Similar increase in blood glucose levels has been reported by Evans *et al*. (2006) following exposure of *O.niloticus* to unionized ammonia. Decreased cholesterol levels were recorded in brook trout post infection with *A. salmonicida* and Atlantic salmon infected with *Vibrio sp*.

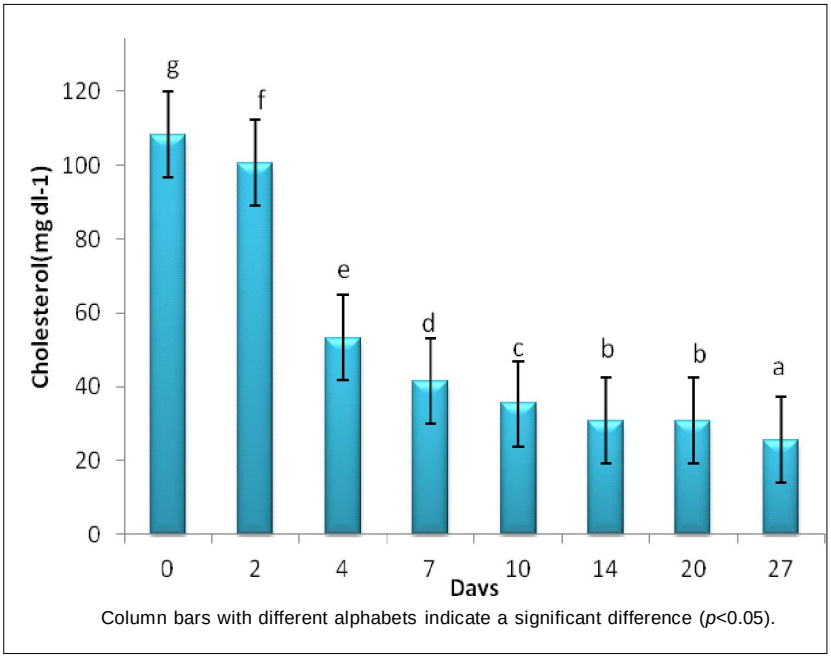
Quantitative real-time PCR (qRT-PCR)

Intraperitoneal injection of *S. putrefaciens* induced a significant increase in the expression of all genes after infection in the samples examined (Fig 3). When compared to the control (1.01±0.1) significantly up-regulated TLR4 (3.64±0.1), GST (4.9±0.3), IL6 (4.41±0.1), HSP70 (3.64±0.1) and HSP90 (3.80±0.1) expression were observed after infection (Fig 4). Similarly, the expression levels of various immune-related genes, namely TLR4, GST, IL6, HSP70, HSP90 in both the spleen and head kidney of mandarin



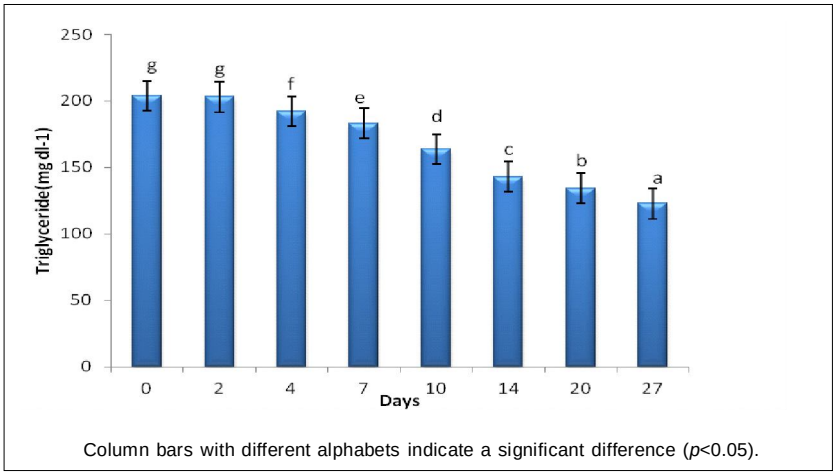
Column bars with different alphabets indicate a significant difference (p<0.05).

Fig 1: Glucose in Nile tilapia, *Oreochromis niloticus* infected with *S. putrefaciens*.



Column bars with different alphabets indicate a significant difference (p<0.05).

Fig 2: Cholesterol in Nile tilapia, *Oreochromis niloticus* infected with *S. putrefaciens*.



Column bars with different alphabets indicate a significant difference (p<0.05).

Fig 3: Triglyceride in Nile tilapia, *Oreochromi sniloticus* infected with *S.putrefaciens*.

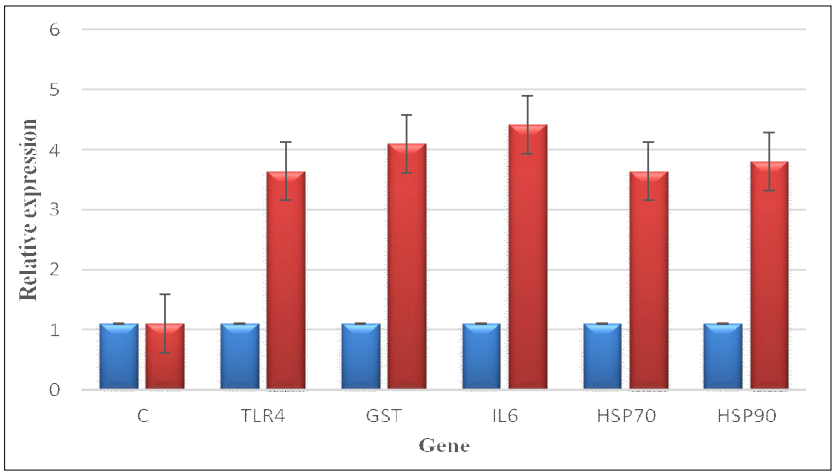


Fig 4: TLR4, GST, IL6, HSP70, HSP90 in Nile tilapia, *Oreochromi sniloticus* infected with *S.putrefaciens*.

fish (Wang *et al.,* 2016). Similarly *A. hydrophila* infected *L. rohita* showed reduced gene expression of HSP70 and HSP90 (Das *et al.,* 2016).

CONCLUSION

To the best of our knowledge, this is the first study to document the hematobiochemical, immune response and immune gene expression of Nile tilapia against *S. putrefaciens* infection. It may be tremendously helpful to the fish farming and large-scale aquaculture industry for diagnostic purpose.

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Conflict of interest

The authors declare that they have no conflict of interest.

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