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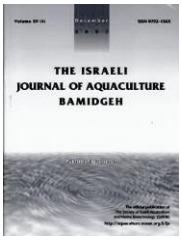

Expression Differences of Stress and Immunity Genes in Rainbow Trout (*Oncorhynchus mykiss*, *Walbaum 1792*) with Different Bacterial Fish Diseases

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Keywords: fish diseases; gene expression; immunity; rainbow trout; antibiotic resistance

Abstract

The aim of this study was to determine the changes in the mRNA transcription levels of HSP70 and IGF genes related to stress and immunity in *Oncorhynchus mykiss* obtained from fish farms, to determine the phenotypic and antimicrobial properties of the bacteria isolated in the study and to compare the results according to different diseases. Accordingly, six fish from each fish farm, a total of 30 fish were sampled. Bacterial identification, inoculations were carried out from the tissue samples taken from the kidney and symptomatic surfaces of fish samples in TSA. Primary cultures were obtained after incubation periods at 21 and 37°C. Gram staining, catalase, and oxidase tests were performed. Forty-six biochemical and 26 antimicrobial tests were performed using BD Phoenix ID Panels. Muscle tissue was used to determine mRNA gene expression differences and the tissues were preserved at -80°C in RNAlater storage solution. Following the RNA isolation and cDNA synthesis, a real-time PCR procedure was performed with b-Actin (ACTB), Insuline-like growth factor (IGF) and HSP70 Gene Primer Array syste*m. Enterococcus faecium (E. faecium), Lactococcus garvieae (L. garvieae*) and *Staphylococcus aureus (S. aureus)* agents were isolated in the study. These bacteria were identified with 91-99% similarity ratios. No disease agents in the gene expressions of the fish samples in were isolated and they were therefore used as controls. Compared to the control group, heat shock protein (HSP) mRNA expression was upregulated in fish tissues infected with *E. faecium*, *S. aureus,* and *L. garvieae* whereas no significant increase was determined in fish tissues infected with *S. aureus*. IGF mRNA gene expression was upregulated in all infected tissues. IGF expression was upregulated at the highest level in fish tissues infected with *L. garvieae*.

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Introduction

Fishery products are important in terms of both employment and nutrition. As the fisheries sector develops in the world, it increasingly brings some important disease-related issues. Both infectious and non-infectious diseases cause economic losses in the fisheries sector and these require prophylactic measures to prevent them spreading. Accordingly, the identification of disease factors, especially early detection in aquaculture farms is of great importance (Çağırgan, 2007).

The most important factor in natural disease control is the immune system of the fish. In nature, the immune system of fish is usually divided into three: epithelial/mucosal barrier, humoral parameters, and cellular components. Epithelial tissues and mucosa of the skin, gills, and feeding tract that are constantly exposed to environments containing potentially harmful agents comprise a highly important disease barrier in fish. In addition to providing physical and mechanical protection, fish mucus contains various immune defense parameters including antimicrobial peptides, complementary factors and immunoglobulins. The immune system protects the body against diseases by identifying and eliminating the pathogen (Magnadottir, 2010).

Antibiotics are often used to treat diseases. However, misuse of antibiotics can cause resistance to pathogens. Although the use of vaccination greatly reduces the use of antibiotics, limited information on the immune system of fish limits the development of new vaccines (Mohanty and Sahoo, 2010). Exposure to biological and abiotic stress factors causes biochemical and physiological changes in the body of fish (Wendelaar Bonga, 1997). It was determined that the response to stress at the cellular level was different in almost all organisms (Feder and Hofmann, 1999). One of the cellular responses to stress, functions within the cell by stimulating a protein family known as heat shock protein (Welch, 1993). In case of infection, the body recognizes these factors and mobiizes defense systems. This increases the level of stress and the immune system activity in the body, resulting in the eventual increase of the transcription activity at the gene protein level (Basu et al., 2001). Some studies on this subject have reported significant relationships between non-specific immune parameters such as disease resistance and bacterial activity in fish (Roed et al., 1992; Marsden et al., 1996; Mohanty et al., 2007; Mohanty and Sahoo, 2010).

In this study, the differences in IGF and HSP gene expression levels were determined in *Rainbow trout* infected with different bacterial disease agents in fish farms from Bayburt, Turkey. The bacteria isolated in this study were identified with 46 different biochemical tests and their sensitivity to 26 different antibiotic substances were examined.

Materials and Methods

Sampling and Experimental layout. This study was carried out with the authorization of the Local Ethics Committee of Animal Experiments of Van Yüzüncü Yıl University on 31.05.2018, with permission No.2018/05. Fish samples used in this study were collected from *Rainbow trout* farms located in Bayburt-Turkey in May-June 2018. Six fish samples were collected from each farm. The collected samples were brought on the same day to the laboratory chilled at 4°C. The study was carried out in Van Yüzüncü Yıl University, Faculty of Fisheries, Disease Laboratories and Biotechnology Application and Research Center Laboratories.

Bacterial Isolation.

Samples taken from the kidney and symptomatic fin tissues of the fish were inoculated into Tryptic Soy Agar (TSA) medium under aseptic conditions. Of the culture bacteria after 48-hour incubation at 21 and 37°C. Colonies with different morphological structures were separately purified and examined with Gram staining, catalase and oxidase tests. According to the Gram staining results, new fresh cultures were obtained and used for the biochemical tests (Gil et al., 2000; Austin et al., 2012).

Bacterial identification and antibacterial activity.

The isolates included a total of 2 mL in a McFarland solution of 0.5 optical density (OD). The bacterial suspensions were inoculated in the Phoenix Automated Microbiology System (Becton Dickinson) Gram-positive identification kit. The inoculant was left to incubate at 37°C for 18 hours. The samples were examined for 26 different antibiotic substances by Minimal Inhibitory Concentration (MIC) and Susceptible, Intermediate, Resistant (SIR) tests (Eigner et al., 2009).

RNA isolation and cDNA synthesis.

Muscle tissues (25 g) taken from the sampled fish were preserved in RNAlater. RNA isolation was carried out with RNeasy Plus Mini Kit in QIACUBE (Qiagen) device according to the manufacturer's instructions. The quality of the isolated RNAs was tested with a nanospectrophotometer (Thermo) at 260 and 280 nm wavelengths. A total of 10µl of 1µl/100ng RNA was prepared for the cDNA production. Then, 2µl GE buffer was added and left to incubate in PCR at 42°C for 5 minutes. The total volume was completed to 20µl by adding 4 µL 5X Reaction Buffer, 1 µL Primer (Primer Array System, UK) and 2µL Reverse Transcriptase Mix. Finally, incubations were carried out at 42°C for 15 minutes and then at 95°C for 5 minutes to activate the reverse transcriptase (Shahi et al., 2018).

RT-qPCR.

The present study was SybrGreen-based and accordingly, RT2 SybrGreen qPCR Master Mix was used. Two target genes (IGF, HSP) and one reference gene Beta-actin (ACTB), a total of three genes were utilized. PCR composition was set to a total volume of 20 µL, comprising 12.5 µL SybrGreen qPCR Master Mix, 1 µL Forward and Reverse Assay Primer, 6.5 µL H2O. Finally, 5 µL cDNA was added. The PCR protocol was optimized first incubation step at 95°C for 10 minutes, and Annealing at 94°C for 15 seconds and 60°C for 30 seconds; the process was carried out with 40 repetitions (Hoseinifar et al., 2017). The Ct values obtained after Real-Time PCR were normalized using the ACTB reference gene as the coefficient change criterium. Real-time PCR data were analyzed according to the ΔΔCT method. All the samples including non-template controls were run in duplicates. b-Actin (ACTB) was the most stable reference gene under exposure of bacterial diseases in the present study. Genbank sequences for respective primers are given in Table 1.

Table 1. Gene Ref Seq Numbers and symbols.

Position Ref Seq Number	Symbol
AF254414	ACTB
LOC110503689	IGF
NM_001124228	HSP70

Statistical analysis.

Each transcript was analyzed on six individuals per each sampling point per each group. The changes of expression levels of HSP70 and IGF genes with bacterial agents and without bacterial agents' samples were calculated by the 2_DDCt method with the formula, F 1/4 2_DDCt, DDCt 1/4 (Ct, target gene_Ct, b-actin) control (Jing et al., 2019). The data analysis report was exported from Gene Globe. The differences were considered significant at $p<0.05$. One-way ANOVA with Duncan test was used to determine whether the results in treatment groups were significantly different from those of control. The level of significance was determined as $p<0.05$ (Yang et al., 2013).

Results

The average weight of fish samples was determined between 280 and 300 g. The water resources of the Farms I and V were the same. No bacterial growth was observed in the samples collected from these farms. *Enterococcus faecium* was isolated from Farm II, while *L. garvieae* was isolated from Farm III and *Staphylococcus aureus* was isolated from Farm IV. The owners of the Farms II, III and IV have reported that they provided the fry fishes from different farms. Gram staining, catalase and oxidase tests were carried out on the isolated agents (Table 2).

Table 2. Some microbial test results of isolates

Agents	*Gram strain*	*Oxidase test*	*Catalase*
Staphylococcus aureus	+	-	+
Lactococcus garvieae	+	-	-
Enterococcus faecium	+	-	-

As a result of the biochemical tests, the isolated bacteria were identified at 91-99% level. The biochemical test results conducted using the BD Phoenix ID kit are given in Table 3.

Table 3. Biochemical test results of isolated bacteria

Biochemical Tests	*Substrate Name of Tests*	*Staphylococcus aureus*	*Lactococcus garvieae*	*Lactococcus garvieae*	*Enterococcus faecium*
A_ARARR	ARGININE-ARGININE-AMC	+	-	-	-
A_LARGH	L-ARGININE-AMC	+	-	-	-
A_LLEUH	L-LEUCINE-AMC	-	+	+	-
A_LPYR	L-PYROGLUTAMIC ACID-AMC	-	+	+	-
C_3MGA	3-METHYL GLUTARIC ACID	+	-	-	-
C_DGUA	D-GLUCONIC ACID	+	+	+	+
C_KGA	ALPHA-KETOGLUTARIC ACID	+	-	-	+
C_THY	THYMIDINE	+	+	+	+
M_BDGAL	4MU-BD-GALACTOSIDE	-	-	-	-
M_NAG	4MU-N-ACETYL-BD-GLUCOS AMINIDE	-	-	-	-
N_ALALH	ALANINE-ALANINE-PNA	+	+	+	-
P_ADGLU	4MU-AD-GLUCOSIDE	-	+	+	-
R_DEX	DEXTROSE	+	+	+	+
R_DTRE	D-TREHALOSE	+	+	+	+
R_NGU	N-ACETYL-GLUCOSAMINE	+	+	+	+
R_MPG	METHYL-ALPHA-D-GLUCOPYRANOSİDE	-	-	-	-
A_GLPRB	GLYCINE-PROLINE-AMC	-	+	+	-
A_LHIST	L-HISTIDINE-AMC	-	-	-	-
A_LPHET	L-PHENYLALANINE-AMC	-	+	+	+
A_LTRY	L-TRYPTOPHAN-AMC	-	+	+	+
C_CLST	COLISTIN	-	+	+	+
C_DMNT	D-MANNITOL	+	+	+	-
C_MAA	3-METHYLADIPIC ACID	+	-	-	-
M_ADGLU	4MU-AD-GLUCOSIDE	-	+	+	-
M_BDGLC	4MU-BD-GLUCURONIDE	-	-	-	-
M_PHOS	4MU-PHOSPHATE	-	-	-	-
N_LPROT	L-PROLINE-PNA	-	-	+	-
P_PHOL	PNP-PHOSPHATE	-	-	-	-
R_DSUC	D-SUCROSE	+	+	+	+
R_MAL	MALTOSE	+	+	+	+
S_URE	UREA	-	-	-	-
A_LALT	L-ALANINE-AMC	+	+	+	-
A_LISO	L-ISOLEUCINE-AMC	-	-	-	-
A_LPROB	L-PROLINE-AMC	-	-	-	-
A_META	METHIONINE-AMC	-	+	+	-
C_DFRU	D-FRUCTOSE	+	+	+	+
C_IMN	MINODIACETIC ACID	-	-	-	-
C_PXB	POLYMYXIN B	-	+	+	+
M_BDCEL	4MU-BD-CELLOBIOSIDE	-	+	+	+
M_BDGLU	4MU-BD-GLUCOSIDE	-	+	+	+
M_PHOT	4MU-PHOSPHATE (with Trehalose)	-	-	-	-
N_VAALA	VALINE-ALANINE-PNA	-	+	+	-
R_BGEN	BETA-GENTIOBIOSE	-	+	+	-
R_DTAG	D-TAGATOSE	-	+	+	-
R_MTT	MALTOTRIOSE	+	+	+	+
T_ESC	ESCULIN	+	+	+	+

As a result of the antimicrobial tests in BD Phoenix ID microbiological system, it was found that *E. faecium* was sensitive to Gentamicin-Syn, Streptomycin-Syn, Ampicilin, Daptomycin, Teicoplanin, Vancomycin, Erytromycin, Quinupristin-dalfopristin, Linezolid, Nitrofurantoin, Ciprofloxacin, Rifampin and Tetracycline and intermediately sensitive to Levofloxacin. It was also found that the species was resistant to Gentamicin, Tobramycin, Cefoxitin, Trimethoprim-Sulfamethoxazole, Clindamycin, and Fusidic Acid. It was found that *S. aureus* was sensitive to Gentamicin, Tobramycin, Trimethoprim-Sulfamethoxazole, Ciprofloxacin, Levofloxacin and Tetracycline whereas it was resistant to Ampicilin, Penicilin G, Oxacillin, Amoxicilin-Clavulanate, Quinupristin-dalfopristin, Linezolid and Nitrofurantoin. *L. garvieae* was found to be sensitive to Gentamicin-Syn, Streptomycin-Syn, Ampicilin,Teicoplanin, Vancomycin, Erytromycin, Quinupristin-dalfopristin, Nitrofurantoin, Ciprofloxacin, Levofloxacin and Tetracycline, and resistant to Gentamicin, Tobramycin, Cefoxitin, Penicilin G, Amoxicilin-Clavulanate, Trimethoprim-Sulfamethoxazole, Clindamycin, Rifampin and Fusidic Acid (Table 4).

Table 4. MIC and SIR values of isolates in this study.*

Antibiotics	*E. faecium*		*L. garvieae*		*S. aureus*	
	MIC	SIR	MIC	SIR	MIC	SIR
Gentamicin-Syn	<=500	S	<=500	S	<=500	X
Streptomycin-Syn	<=1000	S	<=1000	S	<=1000	X
Gentamicin	4	R	4	R	2	S
Tobramycin	4	R	>4	R	2	S
Cefoxitin	>8	R	>8	R	8	X
Ampicilin	<=2	S	<=2	S	>8	R
Penicilin G	>0.25	X	>0.25	R	>0.25	R
Oxacillin	>2	X	>2	X	>2	R
Amoxicilin-Clavulanate	<=2/1	X	<=2/1	R	<=2/1	R
Daptomycin	2	S	1	X	>4	X
Trimethoprim-Sulfamethoxazole	<=0.5/9.5	R	>2/38	R	<=0.5/9.5	S
Teicoplanin	<=0.5	S	<=0.5	S	>4	X
Vancomycin	1	S	<=0.5	S	>4	X
Clindamycin	<=0.25	R	>1	R	>1	X
Erytromycin	<=0.25	S	<=0.25	S	>2	X
Quinupristin-dalfopristin	<=0.5	S	>2	S	>2	R
Fusidic Acid	2	R	>8	R	>8	X
Linezolid	2	S	X	X	>4	R
Fosfomycin w/G6P	>64	X	>64	X	<=16	X
Nitrofurantoin	32	S	32	S	>64	R
Ciprofloxacin	<=1	S	<=1	S	<=1	S
Levofloxacin	4	I	<=1	S	<=1	S
Rifampin	<=0.25	S	>1	R	>1	X
Tetracycline	<=0.5	S	<=0.5	S	<=0.5	S
Tigecycline	<=0.25	X	<=0.25	X	1	X

* S; Susceptible, R; Resistant, X; No-Result, I; Intermediate, MIC; Minimum inhibitory concentration, SIR; Susceptible-Intermediate-Resistant.

Compared to the control group in which no disease agent was isolated, mRNA expression for IGF gene was upregulated at the highest levels in the fish tissues obtained from samples collected from Farm IV in which *L. garvieae* was isolated. This was followed by the mRNA expression upregulation levels for IGF gene in samples collected from Farm III in which *E. faecium* was isolated and in samples collected from Farm V in which *S. aureus* was isolated. HSP gene mRNA gene expression levels were the highest and significantly upregulated in fish samples infected with *L. garvieae* isolated fish samples, whereas no significant upregulation was observed in tissue samples infected with *E. faecium* and *S. aureus* (Fig. 1). No significant changes were observed in the mRNA levels in terms of HSP70 and IGF gene expression in fish samples collected from Farms II and VI. This is likely because the sampled fish had approximately the same size and were not

exposed to the disease agent. Because, the genes examined in the study are more affected at the level of expression by other environmental conditions, especially in disease situations. Moreover, the facts that the waters in the farms had the same quality and the place where the fish were purchased were the same supported these results.

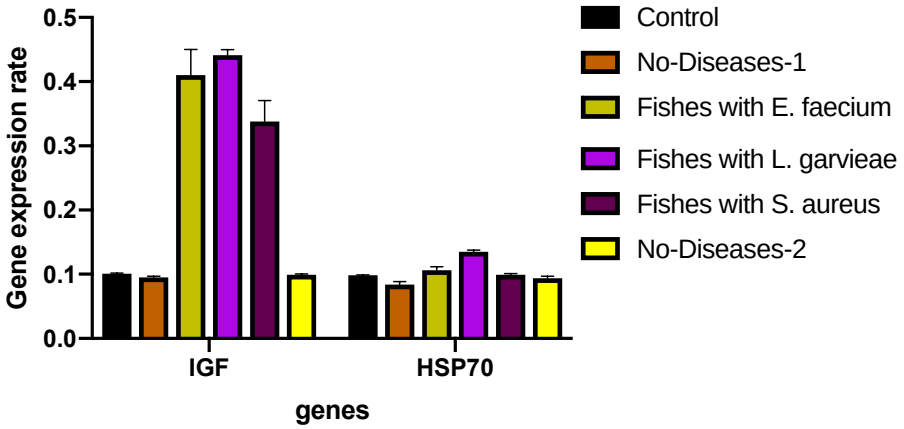

Fig. 1. mRNA transcript levels of IGF and HSP genes in *Rainbow trout* muscle tissues (The values represent mean ± SD of three independent samples; Error bars indicate standard deviation. Statistically significant differences (* P<0.05, ** P<0.01, *** p<0.001) were analyzed by One-Way ANOVA).

Discussion

Bacterial disease agents differ in their effects on the host body. *L. garvieae*, one of the bacterial species isolated in this study, is known as a fish pathogen that especially affects rainbow trout, Japanese yellowtail, and grey mullet (*Mugil cephalus*) species (Zuily, 2011). *E. faecium*, classified as Enterococcus, is a Gram-positive bacterium which can be alpha-hemolytic or non-hemolytic (Ryan and Ray, 2004). The most important factor affecting its microbial growth is temperature. The optimum development temperature is 37°C (Chakraborty et al., 2019). It was also reported that this agent also affects growth and immune system (Supamattaya et al., 2005). Staphylococcus species are Gram-positive cocci that cause infections in fish. It has been reported that it causes significant economic losses in the fishery industry (Kim et al., 2008). Assessing the antibiotic susceptibility of Methicillin Resistant *S. aureus* bacteria (MRSAs), it was found that they had developed sensitivity to vancomycin and teicoplanin, high sensitivity to sulfamethazine/trimethoprim (88.2%), low sensitivity to oxacillin, clindamycin, penicillin and erythromycin (Park et al., 2008). PCR detection of these three bacterial species was carried out in some studies. It was also reported that *E. faecium* was isolated from water, sediments and trout samples (Novais et al., 2013). Mortality rates also vary in the same fish species that are exposed to different disease agents. Therefore, different disease agents cause different levels of gene expression associated with growth and immunity in the fish they use as hosts. In this study, the characteristics of the bacterial agents isolated from rainbow trout obtained from different fish farms were investigated and the differences in the expression of HSP and IGF genes in these fish samples were examined. As a result of 56 different tests examining the biochemical properties of *L. garvieae*, *E. faecium,* and *S. aureus* isolates, it was determined that two different *L. garvieae* isolates had similar biochemical properties, *E. faecium* had the closest biochemical properties to those of these two isolates and the biochemical properties of *S. aureus* isolates substantially differed from those of the other bacteria.

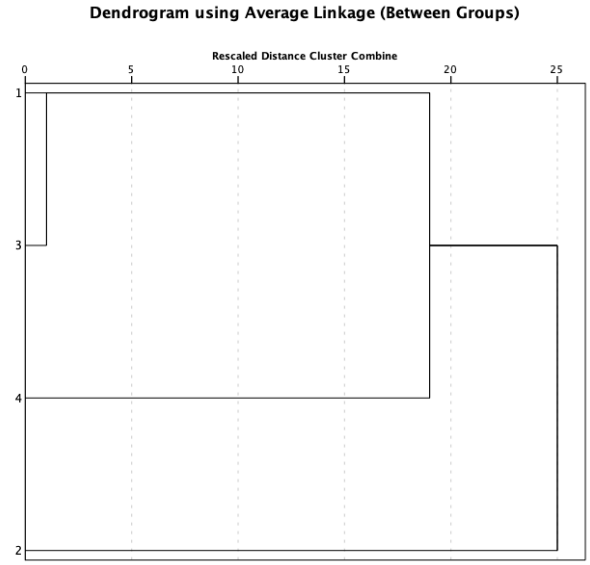

Fig. 2. Biochemical differences between bacterial isolates in this study (1-3; *L. garvieae*, 2; *S. aureus*, 4; *E. faecium)*

Gene expression differences were determined from RNAs isolated from muscle tissues of rainbow trout samples exposed to different disease agents. In the previous studies, it has been reported that the relative expression ratios obtained from muscle tissue were the highest (Shahi et al., 2018; Furlan et al., 2018). In another study on rainbow trout, it was found that, among the disease agents *A. salmonicida*, *L. garvieae*, *Y. ruckeri,* and *F. psycrophilium*, *Y. ruckeri* yielded the highest upregulation levels in disease induced gene expression, followed by *L. garvieae* (Furlan et al., 2018). Since *Y. ruckeri* was not one of the agents isolated in the present study, no comparison could be made with the literature. However, *L. garvieae* was the agent that exhibited overexpression compared to the other agents isolated.

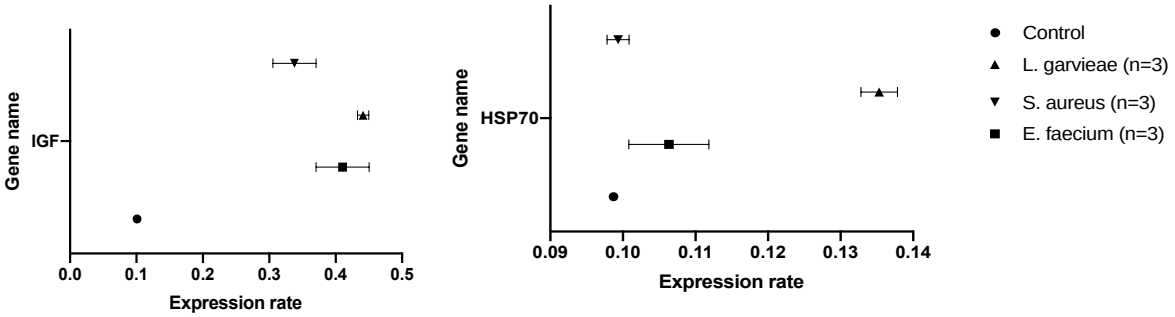

Fig. 3. Overexpressed levels according to different diseases agents (IGF=Insulin growth factor, HSP70=Heat shock protein) (IGF Control: 1.010, HSP Control: 0.0987).

As seen in Figure 3, the highest IGF gene overexpression rates in the fish exposed to *E. faecium*, *L. garvieae* and *S. aureus* infections were determined in the fish exposed to *L. garvieae*, with 4.4-fold that of the control. This was followed by *E. faecium* infection with 4.1-fold and *S. aureus* infection with 3.4-fold. *L. garvieae* was found to be the most effective pathogen on growth and stress with 1.37-fold that of the control in terms of the expression levels of HPS gene, which is the other comparison parameter used in the study. This was followed by *E. faecium* with 1.07-fold and *S. aureus* with 1.00-fold. Growth hormone has no direct effect on some cells. It shows its metabolic and physiological effects through the peptides called somatomedin. These polypeptide components are structurally similar to insulin in terms of binding to proinsulin and insulin receptors. IGF gene has been reported to be the most important somatomedin (Yilmaz,

1999). The growth hormone exerts its effect through the locally produced IGF in tissues, and the growth hormone cannot show its effect directly without the presence of IGF. External IGF factor injection has also been shown to increase salt tolerance in Rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Killifish (Mancera and McCormick, 1998). The combined use of HSP and IGF genes in this study makes the results more important since these genes also affect each other. The IGF ship has also been reported to provide a paracrine effect on the carrier effect of gill and kidney epithelium in fish (Sakamato et al., 2005; Yang et al., 1999). Some studies reported that HSP and IGF genes interact at the cellular level in defense and stress situations (Basu et al., 2001; Galt et al., 2018). It has been reported that, in fish, growth and immunity related GH, HSP and IGF gene expressions occur in muscle tissues (Vélez et al., 2017; Galt et al., 2018). *L. garvieae* showed higher effect on fish than the other agents isolated. It is understood that a connection can be detected between the virulence factors and immunity if the present study, which was carried out at HSP and IGF gene levels, can be expanded with different disease agents and gene additions.

Like all living things, fish have been reported to be vulnerable to most infections and produce immune responses at varying levels and have different susceptibility to destroy the pathogen or limit its ability (Wiegertjes et al., 1996). Furthermore, it has been reported that different bacterial agents had an increased rate of gene expression at different levels for parameters such as growth, stress, and immunity of fish in different tissues. It has been reported in the same study that the expression rate varied in genes in immunized and non-vaccinated fish and there was a lower need for the expression of the genes responsible for immunity in the vaccinated groups (Harun et al., 2011). In a study conducted with probiotic bacteria on IGF gene expression levels in *L. garvieae* infection, it was found that *L. garvieae* affected the immune system at a high level, in parallel with the results obtained in this study. However, probiotics caused a decrease in this expression rate. It has been reported that protection against the pathogen can be provided with the stimulation of immunity parameters by probiotics (Mohammadian et al., 2019). Another factor that plays a role in the defense of the body in the presence of pathogens and changes in expression levels of related genes in fish is nutrition and environment. Strong immune system and adequate nutrition play a positive role in the defense mechanism in fish diseases effectively. In a study on low and excessive diet, it has been reported that cholesterol levels and nutrition had an effect on the expression levels of genes that play a role similar to that of IGF (Wang et al., 2018). Another factor that affects stress, growth and immunity in aquaculture species such as rainbow trout is handing the fish. In another study performed in rainbow trout, it has been reported that, in the same way as in the method used in this study, the elimination of stress-growth and immunity-related genes caused an increase in gene expression levels (Krasnov et al., 2005). As a result of this study, in rainbow trout samples of the same species and size infected with 3 different factors, it was found that *L. garvieae* infection was the most effective on mRNA level of the IGF gene, which indirectly affects the growth of fish and is responsible for immunity, and that the other disease agents caused at least two-fold higher overexpression. It was also understood that the effects of different genes on bacterial disease agents can be brought into consideration in terms of their mechanism of action and the development of molecular techniques in combating diseases thus some novel solutions can be suggested. Considering the role of growth, stress, and immunity in diseases in the fish health, it is thought that fish handing, water quality criteria, nutrition, and vaccination are of great importance, and similar studies should be carried out creating these parameters experimentally.

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