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The expression levels of virulence and antibiotic resistance genes in *Lactococcus garvieae* exposed to different conditions

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ABSTRACT

Lactococcus garvieae is the causative agent of lactococcosis, haemorrhagic septicaemia of fish. Lactococcosis causes significant economic loss in aquaculture because it affects many commercially important freshwater and marine species. In this study, it was observed that the bacteria used in the study were identified at a rate of 92.5% in biochemical tests. It was also observed that the *L. garvieae* agent used in the study was resistant to Gentamicin, Tobramycin, Cefoxitin, Penicillin G, Amoxicillin-Clavulanate, Trimethoprim-Sulfamethoxazole, Clindamycin, Fusidic Acid and Rifampin. When the optimum temperature growth of *L. garvieae* agent was taken at 21°C for the control group, virulence gene expression level was up-regulated at temperatures of 25 and 37°C, which is in the range where the agent can grow best, while it was down-regulated at 15 and 45°C. Similarly, it was observed that the gene expression level was significantly up-regulated in bacteria that grew at the nearest temperature to 25°C in the expression level of antibiotic resistance gene compared to the 21°C control group, and the expression levels of bacteria growing at other temperatures were lower. Considering these results, it is understood that the effect of temperature on virulence is significantly effective because the bacteria used in the study was isolated from rainbow trout.

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Introduction

Bacteria have to adjust their physiology according to the host temperature; that means, they manage the induction of both metabolic and virulence gene expression. The presence of virulence factors in bacteria is necessary for the process of infection, even if it is not in the planktonic state. They save energy by not expressing virulence genes until the host feels they are entering the environment (1).

Water temperature is an important environmental stress factor in outbreaks of most bacterial fish diseases in fish farms. Cold water vibriosis, columnaris disease (*Cytophaga columnaris*) and cold-water disease (*Cytophaga psychrophila*) situation outbreaks occur when the water temperature drops to a certain value. Outbreaks in other diseases, such as lactococosis, are related to an increase in water temperature. Interestingly, bacterial diseases, especially in freshwater, occur at temperatures below optimal for the growth of bacteria that cause infection. For example, for *Yersinia ruckeri* the optimum growth temperature is 28°C, while the temperature caused by the disease is 18°C (2).

Known virulence regulation mechanisms are alteration of DNA, transcriptional regulation and post-transactional regulation (3). RNA thermometers modulate the translation efficiency of the mRNA of virulence genes in relation to temperature. These are sequences that can form intramolecular stem-loop structures that affect the ribosomal binding region (Shine-Dalgarno sequence). In this way, at the optimum growth temperature of the bacterium, mRNA compensation makes it inaccessible. When the temperature drops, the stem-loop structure is lost with a conformational change at the 5' end of the mRNA, making the ribosome accessible to the Shine-Dalgarno sequence (1).

An example of this regulatory system (Figure 1) *Escherichia coli* code the cold shock protein of *cspA* is the gene (1). It has a variety of functions, such as maintaining other proteins in an active form under cold conditions or facilitating their translation by connecting to specific mRNAs (4).

Lactococcus garvieae is a Gram-positive, catalase-negative, speculative anaerobic, α-hemolytic chain odor known to cause "fatal hemorrhagic septicaemia" and "meningoencephalitis" in humans, animals and fish (6). *L. garvieae* is one of the most important bacterial fish pathogens affecting various wild and farm-grown fish species, especially rainbow trout, but it is also isolated from other animal species such as cows, buffaloes, pigs, wild birds, cats, dogs and horses (5). *L. garvieae* first form clinical mastitis samples, and yellow-tailed *Seriola quinqueradiata* isolated form were found (observed) in the United Kingdom (6). It continues to be seen worldwide with high mortality, especially in rainbow trout.

Bacteria are often exposed to different environmental effects, and one of the important factors affecting bacterial processes is temperature. To cope with temperature changes, bacteria adapt their physiology to the conditions they live in. The temperature adaptation leads to changes in bacterial gene expression and they have developed sensitive regulation systems to modulate the expression of

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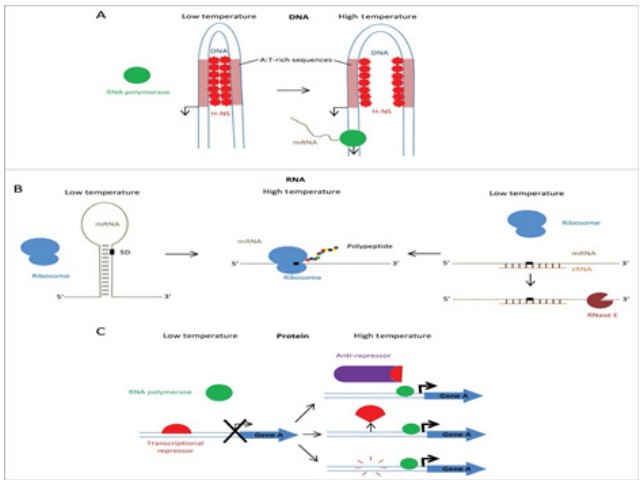

Figure 1. Temperature-related regulator system in bacteria (1).

specific genes. With these regulation systems, they can react quickly to small, moderate or sudden and extreme temperature changes. *L. garvieae* is frequently seen throughout the world and has a high mortality and investigations have been carried out on Biochemical tests of *L. garvieae*, antimicrobial resistance and susceptibility states, expression differences of virulence and antimicrobial resistance genes with temperature changes.

In line with the results of the study, it is thought that the virulence effect in bacterial fish diseases can be determined in aquariums and tanks according to the temperature values by using the data obtained, and mortality in fish populations can be reduced by this way.

Materials and Methods

Bacterial isolation

This study was carried out with the authorization of the Local Ethics Committee of Animal Experiments on 31.05.2018, with permission No.2018/05. The bacteria used in the study were collected from Bayburt (Turkey) province in June 2018. Primary insulations were obtained at the end of 24-hour incubation at 20°C in Tryptic Soy Agar (TSA). Bacteria were kept in culture with 15% glycerol at -80°C until they were used in the study (7, 8). Samples were taken from the kidney and symptomatic tissues of the fish and were inoculated into TSB under aseptic conditions. 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 (9).

Biochemical tests and antibiotic measurement

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. In addition, results from 46 different biochemical tests were obtained from the BD-Phonex system (10).

Incubation of isolates at different temperatures

Isolates developed in the TSA feeder are planted in Tryptic Soy Broth (TSB) (5ml). Incumped for 24 hours a day at 20°C. After the development of bacteria, the suspension solution is adjusted to 0.6 OD at 600nm with a spectrophotometer device (Hach). In the working mechanism, 100 ul (µL) is taken from the same suspension solution for each temperature and inoculated to a 1000ml TSB feeder. In different incubators with incubation temperatures set to 15, 20, 25, 37 and 45°C, bacterial suspensions of the same amount and total volume were incubated for 24 hours a day. After the incubation period, the samples were kept at +4°C. Samples for gene expression analysis were used on the same day (11).

Gene expression analysis

Bacterial RNA isolation and cDNA synthesis

Bacterial 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 nano spectrophotometer (Thermo) at 260 and 280 nm wavelengths. A total of 10µl of 1µl/100ng RNA was prepared for the cDNA production. Then, the 2 µl GE buffer was added and left to incubate in PCR at 42 °C for 5 minutes. The total volume was completed to 20 µl by adding 4 µL 5X Reaction Buffer, 1 µL Primer (Primer Array System, UK) and 2 µL 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 (12).

Real-Time PCR analysis

The present study was SybrGreen-based, and accordingly, RT2 SybrGreen qPCR Master Mix was used. Two target genes (TET, FBP) and one reference gene (GAP), 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 considered. In PCR analysis; GAP primers (5'- TATCGGTCGTCTTGCTTTCC-3', 5'- TCAGCCCAGTTGATGTTAGC-3') were used as controls (13). TET gene primers (5'- ATCAAGATATTAAGGAC-3', 5'- TTCTCTATGTGGTAATC-3') were used for antimicrobial resistance gene expression. FBP gene primers (5'- CGGTTCGTTCAGGAAGAACATC-3', 5'- CGGTCATTGCCTACTTGCTCAA-3') were used for virulence gene expression. The PCR protocol was optimized for the first incubation step at 95°C for 10 minutes and annealing at 94°C for 15 seconds and at 60°C for 30 seconds; the process was carried out with 40 repetitions (13). The Ct values obtained after Real-Time PCR were normalized using the GAP reference again as the coefficient change criterium. Real-time PCR data were analyzed according to the ΔΔCT (the delta-delta CT) method. All the samples including non-template controls were running in duplicates. GAP was again the most stable reference under exposure to bacterial diseases in the present study.

Statistical analysis

Each transcript was analyzed as 3 repetitions within its group. In different temperature groups (15, 20, 25, 37, 45°C) changes in expression levels of TET and FBP genes were calculated with the formula 2^DDCt (Ct, target gene Ct, GAP) (14). The data analysis report was exported from Gene Globe. The differences were considered significant

at $p<0.05$. One-way ANOVA with the Duncan test was used to determine whether the results in treatment groups were significantly different from those of the control. The level of significance was determined as $p<0.05$ (15).

Results

Bacterial isolation

After 24 hours at 21°C incubation of bacterial isolates was found to produce Gram-positive, catalase and oxidase-negative results. In different temperature groups (15, 20, 25, 37 and 45°C) bacteria have been observed to form density in all groups in TSB.

Biochemical test results

As a result of biochemical tests performed with the BD-Phonex ID system, it was obtained from BD-Phonex reports that *L. garvieae* insulated 92% above the *L. garvieae* isolate used in the study. The biochemical test results conducted using the BD Phoenix ID kit are given in Table 1.

Antimicrobial test

As a result of the antimicrobial tests in BD Phoenix ID microbiological system, it was found that *L. garvieae* was observed 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, Penicillin G, Amoxicilin-Clavulanate, Trimethoprim-Sulfamethoxazole, Clindamycin, Rifampin and Fusidic Acid (Table 2).

Gene expression analysis

As a result of Real-Time PCR analysis, PCR results obtained by giving sigmoidal curves of samples in all genes are given below. In Real-Time PCR analysis, the automatic threshold value is 0.03. The cut of ct value was determined as 33 Ct during the analysis phase (Figure 2). Samples with the amount of copy DNA generated from *L. garvieae* isolate were used as Standard samples in Real-Time PCR analysis. In this way, differences in the number of PCR amplitude copies of isolates that develop at different temperatures in the housekeeping gene and target genes were also revealed (Figure 2).

In the Real-Time PCR Standard Curve analysis performed with the housekeeping gene (GAP), R^2 with 3 standard samples 0.99933 and efficiency value 0.82, it was ob-

Table 1. Biochemical test results of *L. garvieae* isolates used in the study.

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

Table 2. MIC and SIR values of *L. garvieae* isolate in this study*.

Antibiotics	*L. garvieae*		Antibiotics	*L. garvieae*	
	Mic	Secret		Mic	Secret
Gentamicin-Syn	<=500	S	Vancomycin	<=0.5	S
Streptomycin-Syn	<=1000	S	Clindamycin	>1	R
Gentamicin	4	R	Erythromycin	<=0.2	S
Tobramycin	>4	R	Quinupristin-dalfopristin	>2	S
Cefoxitin	>8	R	Fusidic Acid	>8	R
Ampicillin	<=2	S	Nitrofurantoin	32	S
Penicillin G	>0.25	R	Ciprofloxacin	<=1	S
Amoxicillin-Clavulanate	<=2/1	R	Levofloxacin	<=1	S
Trimethoprim- Sulfamethoxazole	>2/38	R	Rifampin	>1	R
Teicoplanin	<=0.5	S	Tetracycline	<=0.5	S

* Q; Susceptible, R; Resistant, I; Intermediate, MIC; Minimum inhibitory concentration, SIR; Susceptible-Intermediate-Resistant.

served that the number of PCR copies was higher in equal RNA amounts of bacteria developing at 20, 25 and 37°C.

Virulence gene expression Real-Time PCR

In Real-Time PCR standard curve analysis with virulence-related gene (FBP), R^2 with 3 standard samples 0.99933 and efficiency value 0.82. 20, 25 and 37°C, it was observed that the number of PCR copies in equal RNA amounts of bacteria developing was higher than in other groups (Figure 3).

Antibiotic-resistant gene expression Real-Time PCR results

In the Real-Time PCR standard curve analysis performed with the gene associated with antibiotic resistance gene (TET), R^2 with 3 standard samples 0.99933 and efficiency value 0.82. 20 and 25°C, it was observed that the number of PCR copies in equal RNA amounts of bacteria developing was higher than in other groups (Figure 4).

When the optimum temperature of *L. garvieae* is taken as a control of 21°C, it was observed that there was up-regulation at the virulence gene expression level at temperatures of 25 and 37°C, which are within the range where the factor can develop best, while down-regulated at temperatures of 15 and 45°C. Similarly, according to the 21°C-control group, it was observed that the level of gene expression in bacteria that develop at the nearest temperature level of 25°C at the antibiotic resistance gene expression level was significantly up-regulated, and the expression levels of bacteria developing at other temperature levels were low (Figure 5).

When examined for changes in different gene levels at the same temperature, 20°C. It was observed that the expression rates of *L. garvieae* isolates developing in were high in all samples. This is because it is normal for the reference gene to be optimally high in all gene groups. On the other hand, it was observed that there was a significant increase in isolates developing at a temperature of 25°C

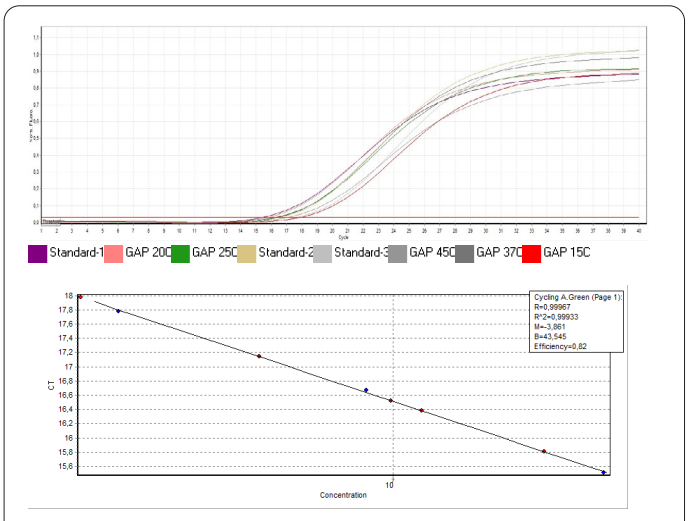

Figure 2. Housekeeping gene expression rates and standard curve analysis results.

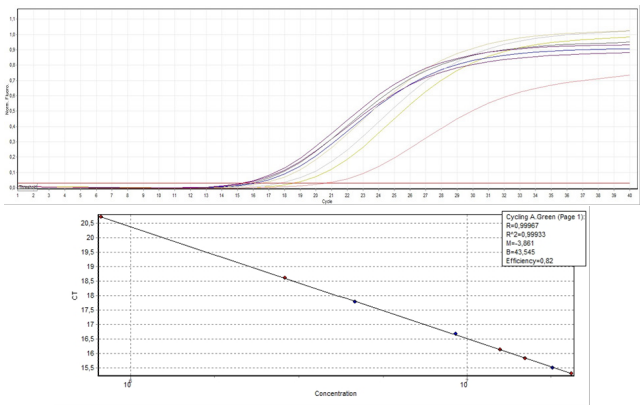

Figure 3. Virulence gene expression rates and standard curve analysis results.

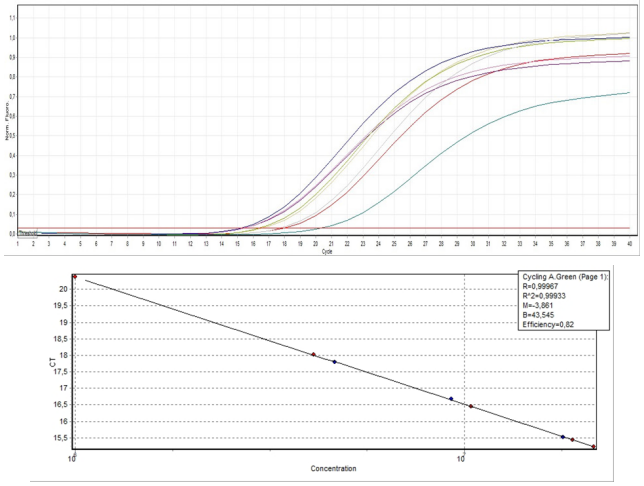

Figure 4. Antibiotic-resistant gene expression rates and standard curve analysis results.

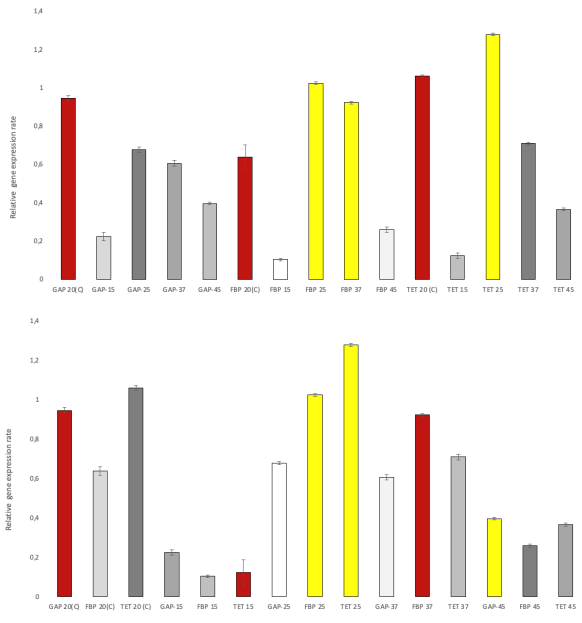

Figure 5. mRNA transcript levels of TET and FBP genes in *L. garvieae* (The values represent mean ± SD of three independent samples; Error bars indicate standard deviation. Statistically significant differences (P<0.05) were analyzed by One Way ANOVA).

compared to the reference gene. This can be explained by the optimum temperature between 21 and 25°C and the higher level of development at these temperatures. This indicates that the bacteria will have higher antibiotic resistance and virulence. In *L. garvieae* factors incubated at a temperature of 15°C, it is understood that expression levels are significantly down-regulated, so the virulence and antibiotic resistance of bacteria developing at these temperatures will also be low. In bacteria that develop at 45°C, it has been observed that the optimum temperature conditions are down-regulation because they are limited at 15°C. Therefore, at this temperature, there is also the development of *L. garvieae* isolates, but it is understood that the system of resistance to medicines and antibiotics will be weak.

Discussion

Many factors determine the virulence and pathogenicity of the bacteria. These are adherence factors, invasion of host cells and tissues, toxins (endo and exotoxins), enzymes (tissue-destroying enzymes, IgA1 proteases), antiphagocytic factors, and antigenic difference (16).

In the worldwide aquaculture industry, despite the great losses caused by *L. garvieae* outbreaks, little is known about the virulence and drug resistance mechanism of this bacterium at the genetic level. Therefore, to understand the molecular mechanism of pathogenicity and drug resistance, the entire genome of *L. garvieae* RTCLI04 has been sequenced by being isolated from clinical specimens of farmed fish rainbow trout in India. According to the sequencing study, the *L. garvieae* RTCLI04 genome is a single circular chromosome of 2,054,885 bp with 39% G+C content. There are 2239 protein-coding genes in the genome. Based on the whole genome analysis of RTCLI04, 51 tRNA genes, 6 rRNA genes (4 genes for 5S rRNA, 1 gene for 16S rRNA, 1 gene for 23S rRNA) are validated and 29 antimicrobial resistance genes and 39 virulence genes (such as hly 1, 2 and 3) have been identified (6).

L. garvieae is a pathogen that affects a wide variety of fish that live from fresh water to marine habitats, causing significant economic losses in aquaculture in the Mediterranean region, Japan, Europe, Southeast Asian countries and North America. In the last few decades, in the summer when the water temperature exceeds 21°C, the "warm-water *lactococcosis*" globally caused by *L. garvieae* has emerged as a major fatal disease of the farmed rainbow trout *Oncorhynchus mykiss*. Because of the lack of appropriate control measures and the increase in internal water temperature due to pathogens and global warming, the movement of the carrier or infected fish from one place to another has caused this disease to spread to pure rainbow trout populations (6).

Despite detailed studies on bacterial ncRNAs, the function of most of them has not been verified. In particular, the role of ncRNAs in virulence regulation is still not fully understood. Currently, only a very small number of ncRNA are specifically illuminated in the regulation of virulence. For example, RNAIII *Streptococcus pneumoniae* has been shown to regulate the expression of multiple virulence genes (17).

Studies on the pathogenesis of *Staphylococcus aureus* have shown that various sRNAs dynamically modulate the synthesis of virulence factors. The most studied sRNA in *S. aureus* is the main intracellular effector of the *agr*-RNA III quorum sensing system agr. That is, RNA III regulates the expression of virulence factors known to be associated with infectious diseases. In other words, RNAIII. Regulates the expression of disease-causing factors known to be linked to infectious diseases. They induce the synthesis of toxins that cause tissue degradation. The latest modelling of the quorum sensing system and regulations has demonstrated the importance of the agr system in promoting the spread of bacteria from biofilms or dense populations. Compared to many clinical isolates obtained from acute infections, higher levels of RNAIII were observed in MRSA strains compared to other *S. aureus* lineages (18).

ncRNAs cover small RNAs (sRNAs) that are widely spread in both prokaryotes and eukaryotes and regulate gene expression. sRNAs have complementary sequences and often form their effects by directly matching bases to other RNA molecules, including mRNAs. Since the ribosomal cannot translate the double-chain RNA, this binding changes the target RNA translation rate. Thus, mRNA of small RNAs constitutes a mechanism in the regulation of the synthesis of already transcribed proteins (4).

RNA molecules that are not transduced to form proteins are called RNA (ncRNA), which are not encoded together. Bacterial ncRNAs are usually small regulatory RNAs (<500 nt). More than 100 ncRAs have been identified in *Escherichia coli* till today. Most chromosome-coded ncRNA has been found to interact directly with target mRNAs, thereby affecting the onset of translation and/or mRNA stability. Generally, a short complementary zone of approximately 7-9 bp is required for ncRNA-mRNA interaction. Although the conventional RNA interference (I) pathway has not yet been defined in bacteria, bacterial ncRNAs have played several key roles in many biological processes by connecting to mRNA targets. Bacterial ncRNAs regulate responses to environmental stresses, including translation control, RNA gradient, and RNA processing. They have been shown to regulate a wide range

of biological processes such as secretion, quorum sensing, stress responses, biofilm formation, adhesion and virulence (17).

The findings of this study show that, *L. garvieae* isolates at 21°C different temperature groups (15, 20, 25, 37 and 45°C) have been observed to develop in all groups in TSB. As a result of biochemical and antimicrobial tests performed with the BD-Phonex ID System, *L. garvieae* identification was performed on 92%. As a result of gene expression analyses, sigmoidal curves were observed in Real-Time PCR analysis in all groups. In standard curve analysis, R^2 with 3 standard samples 0.99933 and efficiency rate of 0.82. 20 and 25°C. It was understood that the number of PCR copies in equal RNA amounts of bacteria developing in other temperatures was higher across the groups than at other temperature levels.

20 and 25°C, which is within the range where *L. garvieae* can develop best. It was observed that there was up-regulation at the level of virulence gene expression at other temperature groups, while down-regulated at 15 and 45°C temperatures. Similarly, the antibiotic resistance gene is at the level of expression of 25°C. It was observed that the level of gene expression in bacteria developing in was significantly up-regulated, and the expression levels of bacteria developing at other temperature levels were low. In line with these results, it is understood that the effect of temperature on virulence is significantly effective because the bacteria used in the study is isolated from rainbow trout. It would be useful to conduct studies on how the same factor being isolated to different species will affect the virulence and antibiotic resistance mechanism.

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Conflicts of interest
There are no conflicts of interest.

Authors' contribution
This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents.

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