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Research article

DETERMINATION OF THE ANTIBACTERIAL EFFECT OF BEE VENOM AGAINST RAINBOW TROUT PATHOGENS AND ANTIBIOTIC RESISTANCE GENE EXPRESSION

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Bee venom (BV) is a rich source of secondary metabolites from honeybees (*Apis mellifera* L.). It contains a variety of bioactive ingredients including peptides, proteins, enzymes, and volatile metabolites. This study investigated the antibacterial effects of the bee venom obtained from honey bees (*Apis mellifera* L.) against bacterial fish pathogens, such as *Lactococcus garvieae* (Lg1, Lg2, Lg3)*, Vibrio anguillarum* (Va1, Va2, Va3)*, Yersinia ruckeri* (Yr1, Yr2, Yr3)*,* and *Aeromonas hydrophila (*Ah1, Ah2, Ah3) and the expression levels on the antibiotic resistance genes *hly* and *fbp (hemolysin* and *fibronectin-binding prot)* of them. It was determined that bee venom had an antibacterial effect against *L. garvieae, L. anguillarum*, and *Y. ruckeri* strains, while it had no effect only against Ah3 and Ah2 bacterial strains. As staded by the gene expression of *hly (hemolysin)* and *fbp* (*fibronectin-binding protein*), among the antibiotic resistance genes the effect levels of bee venom on bacterial species varied, although it affected antibiotic resistance and gene expression level in all bacteria. It was revealed that the expression level was the highest for *V. anguillarum* strains, whereas it was below the control group for *L. garvieae*. i.e the effect of bee venom on the resistance mechanism for *L. garvieae* was much less compared to *V. anguillarum*. Based on the results in the current study it could be concluded that applying bee venom to pathogenic bacteria that cause mortality in the aquaculture sector could induce the defense-related gene and change the broad-spectrum biocontrol activity at the molecular level.

Keywords: *Antibacterial activity, Bee venom*, hly gen expression, Honeybee, Pathogenic fish bacteria

INTRODUCTION

The aquaculture sector is the fastest growing and continuously developing food production sector in the world due to its potential to meet the increasing food demands [1]. This rapid growth process is affected by many adverse conditions,

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such as stress overcrowding, poor water quality, and poor nutrition, at many stages of production [2]. On the other hand, many of the disorders and diseases that are known to occur in fish are the result of the same conditions [3]. Therefore, it can be assumed that there are prerequisites for an increase in the number of pathogenic microorganisms that cause diseases and economic losses in fish [4]. This set of assumptions are confirmed by previous studies of bacterial diseases such as vibriosis, lactococcosis, yersiniosis, Aeromonas septicemia, cold water disease and pasteurellosis which have caused diseases in cultured fish in the whole world [4-7]. Despite the lack of data on antibiotic use in many countries [8,9], the worldwide use of antibiotics is estimated to be between 100-200 thousand tons per year [10,11], and it is reported that approximately 50% of this amount is used for veterinary purposes [12]. Intensive fish farming suggests the development of various bacterial diseases and subsequently increase the use of antimicrobial agents [13]. Although antimicrobials and other drugs are used in aquaculture for prophylactic (protection from disease), therapeutic (treatment) [9,14] purposes, the use of these drugs also brings about many risks for the environment and health safety. Among these risks, the intensive use of antibiotics may lead to the development of resistant bacterial strains [15], and this resistance may even be transferred to other strains, which may result in the failure to fight against diseases [16]. It is known that approximately 20-30% of the antibiotics in the aquaculture sector are used by fish, but the rest contaminates the environment. This contamination may pose a risk to habitats and other living organisms in the aquatic environment [17,18].

Nowadays, there is an increasing need for organic products and bioactive substances that do not leave residues in fish, instead of antimicrobials and drugs, which are synthetic additives used in the aquaculture sector to increase production and fight diseases [19]. It has been reported that various bee-derived products can be used as alternative therapeutic products [21]. In particular, there are various studies on using different bee products such as propolis, royal jelly, and pollen against bacterial agents in aquaculture [20-23]. Today, despite numerous studies on the antibacterial effect of bee venom against bacteria isolated from various sources [24-28], there is a very limited number of studies on using bee venom against bacterial fish pathogens. Although, it has been reported that bee venom has antimicrobial effects against *Edwardsiella tarda, Vibrio ichthyoenteri,* and *Streptococcus iniae* isolated from the intestinal flora of flounder (*Paralichthys olivaceus*) [29], no molecular methods for the expression level of genes that control the virulence and antibiotic resistance system of bacteria treated with bee venom were applied.

The antibacterial effect of bee venom against *L. garvieae, Y. ruckeri, V. anguillarum (syn: Listonella anguillarum),* and *A. hydrophila* strains, which are known as pathogenic bacteria causing high economic losses in rainbow trout production and the effect of bee venom on the antibiotic resistance genes (*hyl* and *fbp*) of these pathogens were investigated for the first time in the present study.

MATERIAL AND METHODS

Bee venom

Bee venom was produced using honey bee colonies (n:15) found on Muş Alparslan University campus according by Güler [30].

GC/MS (Gas Chromatography–Mass Spectrometry)

The GC/MS analysis of the bee venom content was performed in the form of service procurement at Muğla Sıtkı Koçman University.

Pathogenic bacteria strains

A total of 12 strains, including *L. garvieae* (n=3) (Lg1, Lg2, Lg3), *V. anguillarum* (n=3) (Va1, Va2, Va3), *Y. ruckeri* (n=3) (Yr1, Yr2, Yr3), and *A. hydrophila* (n=3) (Ah1, Ah2, Ah3), pathogenic bacteria isolated from diseased rainbow trout on different farms in Fethiye.

Antibacterial activity

Each of the bacterial strains was incubated separately after being inoculated into tubes containing 10 ml of MHB (Mueller-Hinton Broth). After 24 hours of incubation, it was adjusted to 0.5 Mc-Farland standard ($5x10^7$ cfu/ml) and then diluted to $5x10^6$ cfu/ml. Afterward, 300 mg of bee venom was dissolved in 10 ml of sterile distilled water (30 mg/ml) and filtered through a 0.22 μm filter, and a stock solution of 30 mg/ml was prepared. The resulting bee venom stock solution was used in the disc diffusion testin order to determine the MIC and MBC [31].

The disc diffusion method was performed on MHA (Mueller-Hinton Agar) medium, as recommended by the Clinical and Laboratory Standards Institute [32]. Bee venom (20 μl) impregnated on sterile discs with a diameter of 6 mm was placed on the MHA medium inoculated with bacteria grown in the MHB medium using sterile forceps. At the end of the 24-hour incubation period, the diameters of the zones formed in the medium were measured. A disc impregnated with sterile distilled water was used as a negative control, and a commercially available oxytetracycline (30 μg/disc) disc was used as a positive control.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A two-fold dilution was made from a 30 mg/ml stock solution of bee venom as 30, 15, 7.5, 3.75, 1.875, 0.9375, 0.4687, 0.2343, 0.1171, and 0.0585 mg/ml. Then, 100 μL of bacterial culture at a density of $5x10^6$ cfu/ml was added to the wells, and the total volume was adjusted to 200 μl per well. MIC values were recorded as the lowest

concentration of bee venom completely inhibiting bacterial growth. Furthermore, to determine MBC for each bacterium, the MHA surface was cultivated using a 96-thread apparatus, and after 24 hours of incubation, the MBC value was determined as the concentration at which no growth was detected in the TSA (Tryptic Soy Agar).

Gene expression

Classification of bacterial strains

An experimental setup was created in the form of different groups for each bacterial strains used in this study, for which control and bee venom applications were carried out, consisting of *L. garvieae, V. anguillarum, A. hydrophila,* and *Y. ruckeri* strains isolated at different times (Table 1).

Table 1. Grouping of isolates to be used in expression analysis of antibiotic resistance genes

Group-1 *L. garvieae* N=5	Group-3 *Y. ruckeri* N=5	Group-5 *L. anguillarum* N=5	Group-7 *A. hydrophila* N=5
Group-2 *L. garvieae* Control N=5	Group-4 *Y. ruckeri* Control N=5	Group-6 *L. anguillarum* Control N=5	Group-8 *A. hydrophila* Control N=5

Identification of gene targets DNA amplifications

The real-time PCR mix comprised primer pairs (to be used separately for each gene) (2 yl), bacterial DNA (4 yl, SYBRGreen qPCR master mix (12.5 yl), and DNase-Rnase free water (6.5 ul). The PCR cycle was completed after incubation at 94 °C for 2 minutes, at 60 °C for 60 seconds, followed by 35 cycles of 1 minute at 72 °C. The reaction was terminated with a final elongation at 72 °C for 7 minutes [33].

RNA extraction and cDNA synthesis

A total of 4 groups (40 isolates) from each group grown in TSB (Tryptic Soy Broth) for RNA isolation were washed three times with PBS buffer after centrifugation and resuspended in 1.5 mL of RNase-free water (Macherey-Nagel, Düren, Germany). Total RNA was isolated using the RNeasy Mini Kit (Qiagen). In the final step, the RNA was converted to cDNA using the RT^2 First-Strand cDNA Synthesis Kit [34].

Antibiotic resistance gene expression

The gene expression level was determined fluorometrically using the SYBR Green-based SYBRGreen qPCR Master Mix (Qiagen). The content was completed for each sample using 5 μL of cDNA, a master mix containing 7.5 μL of qPCR master mix, 1.78 μL of RNase-free water, and 0.36 μL of each primer (final concentration 300 nmol/L). Real-Time PCR analysis was performed with the Rotor-Gene Q

(9000-5Plex-Hrm). According to the qPCR temperature procedure, elongation was first carried out at 40 °C for 30 seconds, followed by an initial denaturation cycle of 5 min at 94 °C. A fluorescence reading was performed at the end of each amplification. Relative quantification in which the relevant gene expression was normalized to a housekeeping gene using the 2 ΔCT method was first used, and then the results were evaluated for differences in gene expression with 2 ΔΔCT and Fold Change analyses, and the graphical values were obtained [35].

RESULTS

GC/MS (Gas Chromatography–Mass Spectrometry) findings

According to the results of the GC/MS analysis, it was determined that bee venom contains 3,84 Apamine, 11.76 Phospholipas A2 and 51.17 melittin. (Table 2).

Table 2. Contains % amounts in 1g bee venom of the bee venom content obtained from honey bees in this study based on the GC/MS analysis

Bee venom	Apamine %	Phospholipase A2 %	Melittin %
1 gr	3.84	11.76	51.17

Antimicrobial activity findings

According to the disc diffusion method performed to determine the antimicrobial property of bee venom, it was found that *L. garvieae* (Lg1, Lg2, Lg3), *V. anguillarum* (Va1, Va2, Va3), and *Y. ruckeri* (Yr1, Yr2, Yr3) strains formed an inhibition diameter, but only Ah2 and Ah3, which are among *A. hydrophila* strains, did not form an inhibition diameter (Table 3; Figure 1).

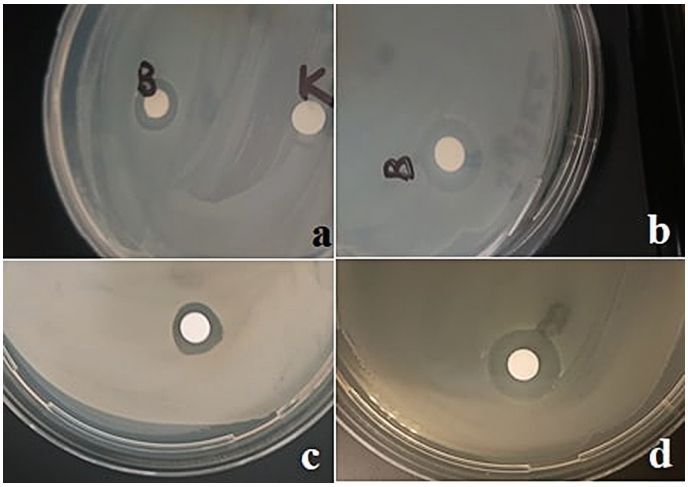

Figure 1. Inhibition diameters of bee venom (30 mg/ml) against pathogenic bacteria **(a)** Va2, **(b)** Yr2, **(c)** Ah1, **(d)** Lg2

Table 3. Zone diameters of bee venom against fish pathogenic bacteria by disc diffusion method

Bacterial codes	Negative control	Positive control (mm)	Bee venom (mm)	Bacterial codes	Negative control	Positive control (mm)	Bee venom (mm)
Va1	-	20	12	Yr1	-	22	16
Va2	-	21	10	Yr2	-	22	15
Va3	-	18	12	Yr3	-	23	15
Lg1	-	10	12	Ah1	-	21	10
Lg2	-	11	16	Ah2	-	20	-
Lg3	-	10	16	Ah3	-	19	-

MIC and MBC analysis findings

Differences in the MIC and MBC values of the strains were detected. The MIC and MBC values acquired are presented in detail (Table 4).

Table 4. Minimum inhibition (MIC) and minimum bactericidal concentration (MBC) values of bee venom

Bacterial strains	MIC value (mg/ml)	MBC value (mg/ml)	Bacterial strains	MIC value (mg/ml)	MBC value (mg/ml)
Va1	0.1171	0.4687	Yr1	0.1171	0.2345
Va2	0.1171	0.2345	Yr2	-	0.1171
Va3	0.1171	0.2345	Yr3	0.0585	0.2345
Lg1	0.1171	0.2345	Ah1	-	-
Lg2	0.0585	0.2345	Ah2	-	-
Lg3	0.1171	0.4687	Ah3	-	-

Antibiotic resistance gene expression analysis findings

The results of the real-time PCR analysis, which was performed with RNA isolations using suspension liquids from each bacterial sample from the experimental groups and then PCR mix using the synthesized cDNA, were obtained in graphs.

In Real-Time PCR analysis, samples above 0.12 automatic Ts value were considered positive, and the gene expression analysis data were interpreted graphically over Ct values. As a result of the real-time PCR analysis, it was revealed that all samples yielded results above the threshold value and all the reagents and components used at the PCR stage were working.

Standard curve analysis was conducted based on the graphic data obtained after real-time PCR analysis, and the results are presented as follows (Figure 2).

After the standard curve analysis, it was confirmed that the R^2 value was appropriate and sample optimization was accurate. Following real-time PCR analysis, before

graphical interpretation, HRM analysis was carried out for the target genes used in the study to confirm the study's accuracy, and the results are given below (Figure 3).

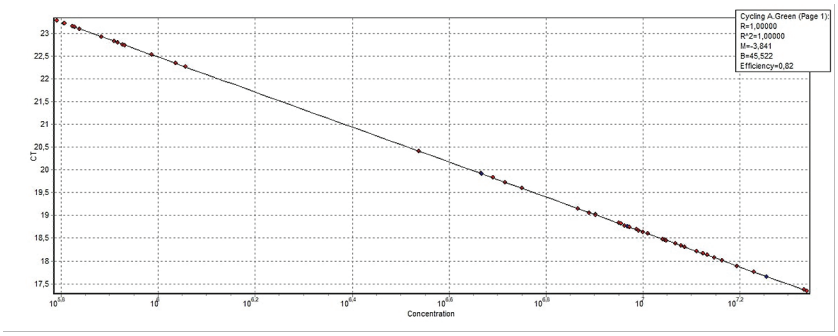

Figure 2. Standard curve analysis graph obtained after Real-Time PCR analysis

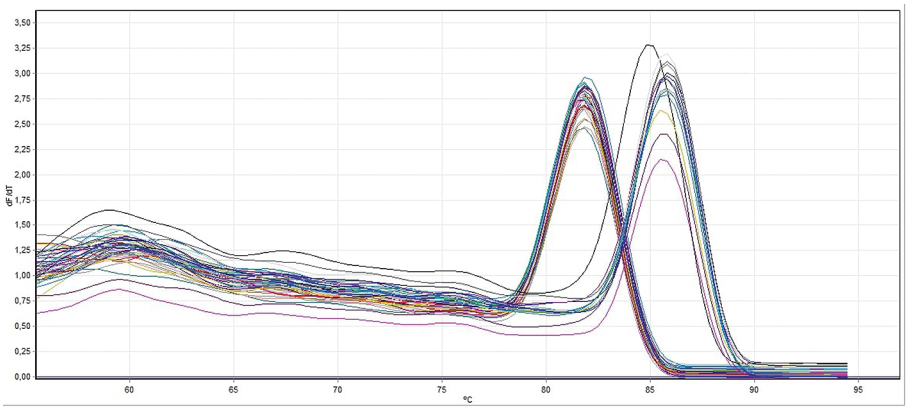

Figure 3. The result of HRM analysis of hyl (hemolysin) and fbp (fibronectin-binding proteins) genes

According to the results of the melting analysis, it was observed that there were two separate peak values for the *hly* (*hemolysin*) and *fbp (fibronectin-binding proteins*) genes. It

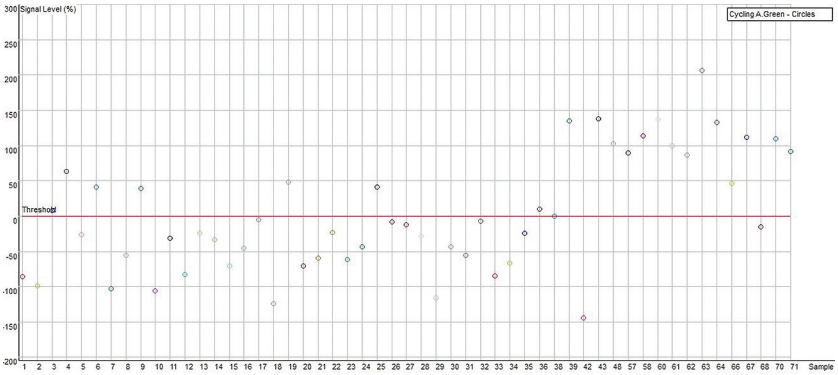

Figure 4. End Point Analysis result of control and target gene samples

was found that the samples were collected on the same peak for each gene (excluding standard, control, and calibrator samples), and the study's accuracy was confirmed. Endpoint analysis results acquired in the gene expression analysis are presented below (Figure 4).

The end point analysis demonstrated that the samples to which bee venom was not applied were below the threshold value and took place as control groups. There was a significant increase above the threshold value in the groups to which bee venom was applied.

In real-time PCR analysis, samples above 0.12 Ts value were considered positive, and the gene expression analysis data were interpreted graphically over Ct values. Real-time PCR analysis showed that all samples yielded results above the threshold value. The relationships between ct values obtained from the graphs and the expressions of genes were obtained with $2^{\Delta\Delta Ct}$ values. Expression levels of antibiotic resistance genes belonging to all groups are given in Figure 5.

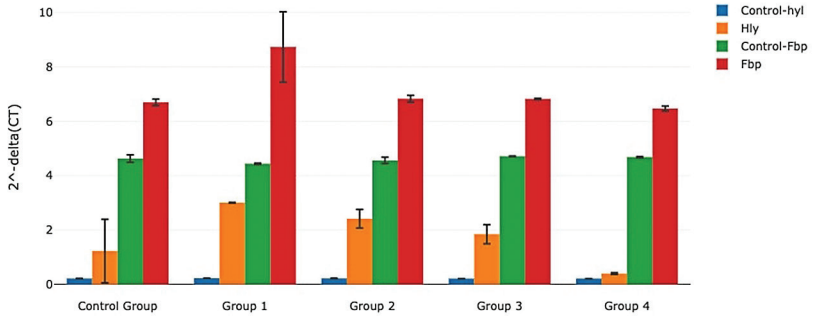

Figure 5. Whole gene-level results of antibiotic resistance gene expression levels *(Control Group: Control groups of all genes, Group 1: V. anguillarum, Group 2: A.hydrophila, Group 3: Y. ruckeri, Group 4: L. garvieae).

Based on the results of the present study, it has been determined that bee venom is effective on antibiotic resistance of all the tested bacteria in relation to the expression levels of the *hly* (*hemolysin*) gene region compared to the control group. The expression

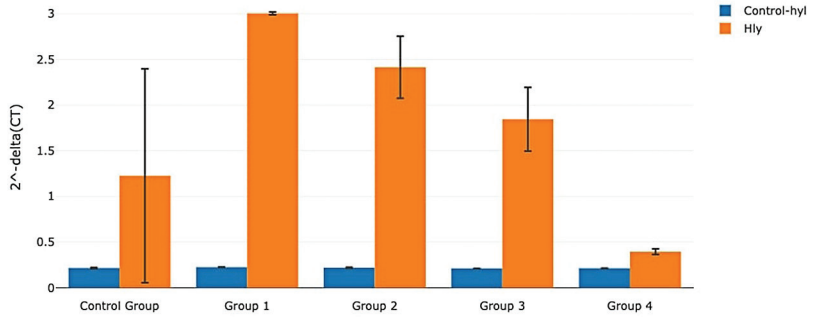

Figure 6. Change of hly (hemolysin) gene expression levels according to groups *(Control Group: Control groups of all genes, Group 1: V. anguillarum, Group 2: A. hydrophila, Group 3: Y. ruckeri, Group 4: L. garvieae)

level of the *hly* (*hemolysin*) gene region in the pathogenic groups used in the study is provided below (Figure 6).

The expression levels of the *fbp* (*fibronectin- binding proteins*) gene region for the study groups are presented in Figure 7.

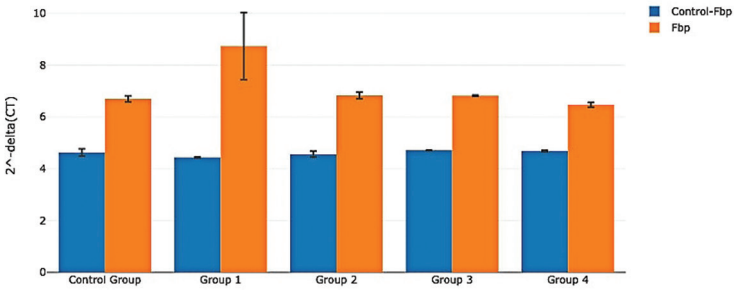

Figure 7. Change of fbp (fibronectin- binding proteins) gene expression levels according to groups *(Control Group: Control groups of all genes, Group 1: V. anguillarum, Group 2: A. hydrophila, Group 3: Y. ruckeri, Group 4: L. garvieae)

The clustergram results for the antibiotic resistance genes in the pathogenic groups and the control groups are presented in Figure 8.

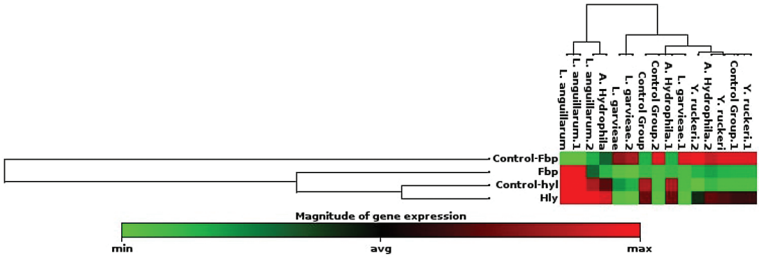

Figure 8. Gene level clustergram profiles of antibiotic resistance genes and control groups

According to the *hly* (*hemolysin*) gene expression results, it was determined that bee venom was effective on antibiotic resistance in all bacteria, based on the control group. However, the effect levels on bacterial species varied. In fact, the expression level was the highest for *V. anguillarum*, whereas it was below the control group for *L. garvieae*. It was considered that the reason for this might be related to the pathogenicity of the bacterium. The effect of bee venom on the resistance mechanism for *L. garvieae*, one of the most effective bacterial agents in the field of aquaculture, was much less compared to *V. anguillarum*.

DISCUSSION

The increase in infections caused by antibiotic-resistant bacteria is shown as one of the most important problems for the healthcare system of the 21st century [36]. Between 2014 and 2016, approximately one million people died from untreated infections

caused by antibiotic-resistant bacteria, which will become very serious for future antibiotic drugs. With the continuation of this situation, project models indicate that there will be an increase in deaths related to antibiotic resistance genes in the next years [37]. Although gene transfer in antibiotic resistance genes is complex, the excessive and frequent misuse of antibiotics in human and veterinary medicine in the European Union is considered the driving force for resistance development. Variants of antibiotic resistance genes have increased and been added to the antibiotic resistance gene pool. Furthermore, the increase in resistant pathogens makes antibiotic treatment difficult [38]. Rich food sources are a factor in the increase in bacterial density. The fact that allochthonous bacteria and pathogenic bacteria are very common is a very important phenomenon in the increase of antibiotic resistance genes [39]. The increase in multi-pathogen resistant bacteria also makes antibiotic treatment difficult. In this process, clinical environments such as repetitive antibiotic treatment and inadequate hygiene are the factors for the increase in antibiotic resistance genes [40]. Due to the antibiotic resistance resulting from unconscious and high amounts of antibiotic use, especially in the aquaculture sector, natural products alternative to antibiotics should be supported by *in vitro* and *in vivo* studies.

Although bee venom has been reported to have antibacterial effects against numerous bacterial agents [41,42], there is no molecular study including the effect of bee venom against pathogenic bacteria causing mortality in fish. In this study, the antibacterial properties of bee venom against bacterial agents such as *L. garvieae, V. anguillarum, Y. ruckeri,* and *A. hydrophila* that cause high mortality in rainbow trout and the changes to be caused by bee venom in the antibiotic resistance mechanism of these bacteria were investigated for the first time at the level of gene expression. It was reported that bee venom had an antibacterial effect against bacteria such as *Edwardsiella tarda, Vibrio ichthyoenteri,* and *Streptococcus iniae* isolated from the intestinal flora of flounder and formed inhibition zones (10.2, 10.1, and 9.5 mm, respectively), and MIC values were 17.6, 0.3, and 3.49 μg/mL, respectively [29]. Since the strains used in this study were isolated from diseased fish rainbow trout on different farms and in different seasons, it was determined that inhibition zones varied between 10-16 mm. Furthermore, it was observed that two *A. hydrophila* strains (Ah2 and Ah3) did not form any zones. It was considered that the antibacterial property of bee venom was due to the active substances in its content (such as melittin, apamin, adolapin, mast cfu degranulation peptide, enzymes, amines, and non-peptide components) [43]. It was reported that bee venom had strong antibacterial properties against Gram-positive bacteria rather than Gram-negative bacterial species [41]. Likewise, in this study, while bee venom did not yield effective results against *A. hydrophila* bacteria, a Gram-negative fish pathogen, it was found to have antibacterial properties *in -vitro* against *L. garvieae*, a Gram-positive fish pathogen.

According to the gene expression results, it was observed that there was a close and insignificant change in the level of regulation in all group samples at the reference gene level. The reason for this was that the control and reference were close to each other

since bacteria did not have any variable factors. This result confirmed the accuracy of the control and reference samples used in the study. As a result of the real-time PCR analysis conducted for gene expression analysis, the automatic threshold value of the samples was found to be 0.12. It was found that all of the PCR amplicons yielded results above the threshold value and all the reagents and components used in the PCR stage were working. According to the results of the melting analysis carried out to confirm the binding accuracy after the PCR analysis, there were two separate peak values for the hly and fbp genes. A single peak result for each gene was considered an indicator of primer-specific binding. The end point analysis demonstrated that samples to which bee venom was not applied were below the threshold value. Hyl gene expression results revealed that bee venom was effective on antibiotic resistance in all bacteria compared to the control group. However, the effect levels on bacterial species varied. The differences in effect according to different bacterial impacts can be listed as follows; the expression level was the highest for *V. anguillarum*, whereas it was below the control group for *L. garvieae*. It was considered that the reason for this might be related to the pathogenicity of the bacterium. It was observed that the effect of bee venom on the resistance mechanism for *L. garvieae*, one of the most effective bacterial agents in the field of aquaculture, was much less compared to *L. anguillarium*.

In conclusion according to the gene expression results for the fbp gene observed in the gene pathway in the antibiotic resistance mechanism, the reference gene was at the same level in all groups and bee venom application caused upregulation in bacteria. However, this rate was found to be low compared to the hyl gene. The reason for this was considered to be the secondary involvement of the gene in the mechanism of action. It was found that *fbp* (*fibronectin- binding proteins*) gene expression created a catalytic effect for *V. anguillarum* at the highest level in cellular response in bee venom application. This study demonstrated that applying bee venom to pathogenic bacteria effective in the field of aquaculture could induce some defense-related genes and change the broad-spectrum biocontrol activity at the molecular level. In our next study, we plan to investigate the efficacy of bee venom at the molecular level as an alternative to antimicrobial products in zebrafish by in *vivo* study.

Authors' contributions

DK designed the study, produced bee venom, and interpreted the data obtained; ÇÜ, contributed to the microbiological analysis and substantial contributions to the conception and design, or acquisition of data, and interpretation of data. ŞÖ, carried out the molecular genetic studies, contributed to gene expression studies and interpretation of results.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ODREĐIVANJE ANTIBAKTERIJSKOG DEJSTVA PČELINJEG OTROVA PROTIV PATOGENA POTOČNE PASTRMKE I EKSPRESIJE GENA REZISTENTNOSTI NA ANTIBIOTKE

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Pčelinji otrov (BV) je bogat izvor sekundarnih metabolita pčela (*Apis mellifera* L.). Sadrži niz bioaktivnih sastojaka uključujući peptide, proteine, enzime i isparljive metabolite. Ovo istraživanje se bavilo ispitivanjem dejstva pčelinjeg otrova dobijenog od pčela

(*Apis mellifera* L.) na bakterijske patogene ribe, kao što su *Lactococcus garvieae* (Lg1, Lg2, Lg3), *Vibrio anguillarum* (Va1, Va2, Va3), *Iersinia ruckeri* (Ir1, Ir2, Ir3) i *Aeromonas hidrophila* (Ah1, Ah2, Ah3) i nivoima ekspresije na genima otpornosti na antibiotike hli i fbp (prot koji vezuje hemolizin i fibronektin). Utvrđeno je da pčelinji otrov deluje antibakterijski na sojeve *L. garvieae*, *L. anguillarum* i *I. ruckeri*, dok nije delovao samo na Ah3 i Ah2 sojeve bakterija. Kao što se vidi po ekspresiji gena hli (hemolizin) i fbp (protein koji vezuje fibronektin), među genima otpornosti na antibiotike nivoi efekta pčelinjeg otrova na bakterijske vrste su varirali, iako je uticao na otpornost na antibiotike i nivo ekspresije gena kod svih bakterija. Utvrđeno je da je nivo ekspresije bio najveći kod sojeva *V. anguillarum*, dok je kod *L. garvieae* bio ispod kontrolne grupe. odnosno efekat pčelinjeg otrova na mehanizam rezistencije za *L. garvieae* bio je mnogo manji u poređenju sa *V. anguillarum*. Na osnovu rezultata u studiji može se zaključiti da primena pčelinjeg otrova na patogene bakterije koje uzrokuju smrtnost u sektoru akvakulture može indukovati gen za odbranu i promeniti aktivnost biokkontrole širokog spektra na molekularnom nivou.