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Araştırma Makalesi (Research Article)

Differences in AChE and BChE Enzyme Activation Levels in Liver and Brain Tissues in Rainbow Trouts Exposed to Different Bacterial Diseases

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Abstract: In this study, symptomatic fish samples were taken from rainbow trout farms. Isolation and identification of agents isolated from fish samples were made. DNA isolations from different purified colonies were carried out with the mericon bacterial DNA kit. Real-Time PCR procedure was performed by using universal bacterial primers. Molecular identifications were performed by blasting the nucleotides obtained by sequence analysis of PCR amplicons. Spectrophotometric measurements were performed at 412 nm wavelengths for AChE activity and 412 nm for BChE activity from liver and brain tissues of fish samples. The activity differences of different disease factors among themselves and according to the control group were examined. As a result of the study, isolation and identification of *Bacillus subtilis, Lactococcus garvieae* and *Staphylococcus epidermidis* from 5 different farms were performed. Over 98% similarity was observed as a result of sequencing analysis of the isolates. In this study, it was observed that three different bacteria isolated from trout farms suppressed AChE and BChE enzyme activities in both tissues of trout.

Farklı Bakteriyel Hastalıklara Maruz Kalmış Gökkuşağı Alabalıklarında Karaciğer ve Beyin Dokularında AChE ve BChE Enzim Aktivite Seviyesi Farklılıkları

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Anahtar kelimeler
AChE,
Bakteri,
BChE,
Dizileme,
Rainbow trout,
Real-Time PCR.

Öz: Bu çalışmada, gökkuşağı alabalığı işlemelerinden semptomlu balık örnekleri alınmıştır. Balık örneklerinden etkenlerinin izolasyonu ve identifikasyonları yapılmıştır. Saflaştırılan farklı kolonilerden DNA izolasyonları mericon bacterial DNA kiti ile gerçekleştirilmiştir. Real-Time PCR prosedürü evrensel bakteriyel primerler kullanılarak gerçekleştirilmiştir. Moleküler identifikasyonlar PCR amplikonlarının dizi analizi ile elde edilen nükleotidlerin blastlaması ile gerçekleştirilmiştir. Balık örneklerinin karaciğer ve beyin dokularından AChE aktivitesi için 412 nm ve BChE aktivitesi için 412 nm dalga boylarında spektrofotometrik ölçümler yapılmıştır. Farklı hastalık etkenlerinin kendi aralarında ve kontrol grubuna göre aktivite farklılıkları incelenmiştir. Çalışma sonucunda, 5 farklı işletmelerin 3'ünden *Bacillus subtilis, Lactococcus garvieae* ve *Staphylococcus epidermidis* etkenlerinin izolasyon ve identifikasyonları gerçekleştirilmiştir. İzolatların dizileme analizi sonucunda %98'in üzerinde benzerlik gözlenmiştir. Çalışmada, alabalık çiftliklerinden izole edilen üç farklı bakterinin her iki dokusunda da AChE ve BChE enzim aktivitelerini baskıladığı görülmüştür.

1. Introduction

As in the whole world, our country's aquaculture industry has entered a rapid development process and reached a production volume of 276 thousand 502 tons according to 2017 data. The most cultivated species in this production volume was rainbow trout, with 109 thousand 657 tons of production (TOB, 2019). Several factors negatively affect the rapid development process in aquaculture production, and infectious diseases come first. Growing fish intensively with a high population density increases the incidence of infections and causes the disease to spread quickly, settle, and continue for a more extended period. In addition, fish diseases have been reported to be affected by environmental factors, especially changes in water temperature (Balta et al., 2016). Vaccine and antibiotic applications play an essential role in preventing the entry of diseases to enterprises and treating diseases. However, these applications lead to some problems. Hundreds of tons of antimicrobials are used every year to prevent and treat bacterial infections in aquaculture. This situation causes the development of bacteria resistant diseases, accumulation of antibiotics in fish tissues and organs indirectly, and harmful results in terms of the aquatic environment and human health (Balta and Çağırgan, 2007).

External effects on metabolism in all living things cause some changes in enzyme systems. Because, enzymes are sensitive biocatalysts, most of which are in protein structure and carry out biochemical reactions (Massouliè et al., 1992; Chatonnet and Lockridge, 1989). Enzymes regulate the speed of many essential reactions for metabolic pathways in cells and increase the speed of reactions. In the central cholinergic system, it is a complex system in which cell bodies and dendrites operate with various enzymes. The cholinergic system is responsible for the transport of impulses between the central and peripheral nervous system cells. Cholinesterase's acetylcholinesterase (AChE) and butyryl-cholinesterase (BChE) are among the essential enzymes of the cholinergic system (Ryhänen, 1983; Ekholm, 2001). Therefore, it was aimed to determine the changes in the enzymes of the cholinergic system of different bacterial diseases.

2. Material and Methods

2.1. Sampling

The study was carried out with the decision of Van Yuzuncu Yil University, Animal Experiments Local Ethics Committee dated 31.01.2019, and numbered 2019/01. The sampling time of the study covers the date range of May-August 2018. The rainbow trout used in the study were selected from the enterprises that produce fry in Van province. For this purpose, 30 fishes, 6 of which were from 5 different establishments, were purchased. Fish that exhibited symptoms such as slowness in movements, separate swimming, darkening in color, symptoms such as bilateral exophthalmos, and such as swimming disorder, fin melting were selected as external symptoms. After the external surfaces of the sampled fish are disinfected with 70% ethanol, each fish sample is separated in separate locked bags. Fish samples were brought to the laboratory at +4 ºC the same day with rubbermaid with a probe thermometer (Touraki et al., 2012).

2.2. Bacteria isolation

Bacteria isolations and molecular-based studies were carried out in Van Yuzuncu Yil University, Faculty of Fisheries, Disease Laboratory. Enzyme studies were performed in Van Yuzuncu Yil University, Faculty of Science, Biochemistry Laboratory. After necropsy performed under aseptic conditions, plantations were made from kidney and symptom fin tissues to TSA. The media were incubated at 21 and 37 ºC for 24 hours (Austin and Austin, 2016).

2.3. Molecular identification

Real-Time PCR procedure was performed using 27F-1492R universal primers for the identification of bacteria. For this purpose, DNA isolations from bacteria were performed with mericon bacterial DNA kit (Qiagen). The purity of DNA was measured with a nano-

spectrophotometer (Thermo). Real-Time PCR was performed using DNA and universal primers isolated from bacteria (Inoue et al., 2005). DNA amplification was performed with a RotorGene Q 5Plex-HRM (Qiagen) at 95 °C for 10 min, followed by 45 cycles, each one divided into denaturation (94 °C, 45 sec), annealing (56 °C, 30 sec), and elongation (72 °C, 45 sec), plus a final elongation at 72 °C for 7 min (Metin et al., 2014).

2.4. Sequence analysis

After Real-Time PCR procedure performed for bacterial identification, sequence analysis was performed using the Sanger method using PCR amplicons and primer pairs. In line with the sequence data, the similarity rates were evaluated on the NCBI website. Nucleotide sequences are interpreted in CLC Workbench software (Zhu et al., 2014).

2.5. Biochemical analysis

Fish tissue samples were weighed and homogenized with phosphate buffer (50 mM KH2PO4 and 10 mM EDTA). Tissue samples homogenized with the homogenizer are 10 minutes at 3500 rpm centrifuged. After the centrifugation process, the liquid part was taken, and the analyses were done. Acetylcholinesterase and Butyrylcholine esterase levels were determined in the brain's homogenates, and liver tissues were taken from fish. Acetylcholinesterase and Butyrylcholine esterase activity analyses were performed according to the colorimetric method of Ellman et al. (1961). Cholinesterase catalyses the breakdown reaction of acetylthiocholine to thiocholine and acetate. 5-thio-2-nitrobenzoic acids, which turn yellow as a result of the reaction of DTNB with the thiocholine ratio released as a product, is formed. The absorbance values of the prepared mixes were read with a spectrophotometer at a wavelength of 412 nm (Yeltekin and Oğuz, 2018).

2.6. Statistical analysis

The results are expressed as mean ± standard error. The one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were applied to the data, and the differences between the data were determined ($p < 0.05$). SPSS 20 software was used for statistical calculations (SPSS Inc., 2020).

3. Results

3.1. Bacteria isolation

It was observed that the water temperature varied between 15-17 ºC during sampling. Fish samples were selected from fish that were left alone in the aquaculture ponds, floating outside of the flock, darkening in colour, and shaped by exophthalmos in the eyes. In the necropsy procedure performed after sampling, samples were taken from kidney and symptom fin tissues. Planting was performed on TSA and BA media from kidney and symptom fin tissues taken during necropsy. Colonies that developed after a 24-hour incubation period at 37 ºC were purified again. The medium images of the purified isolates are given below (Figure 1).

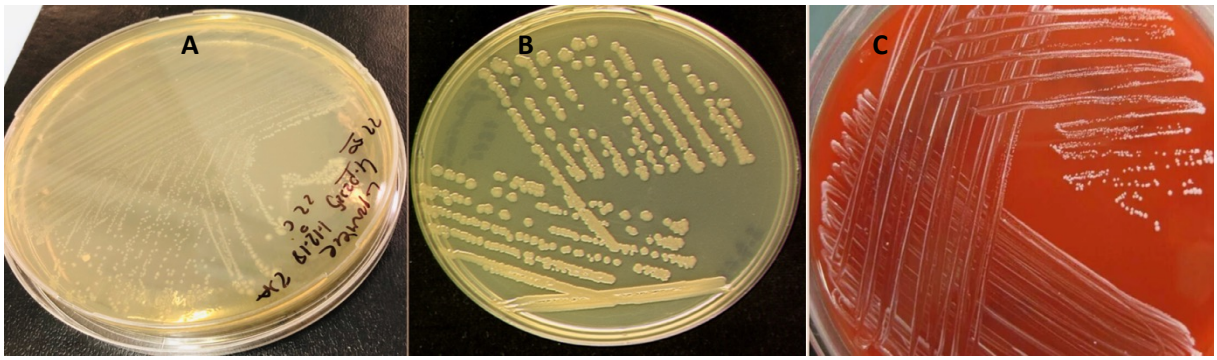

Figure 1. Bacterial disease factors isolated from rainbow trout farms (A: *L. garvieae*, B: *B. subtilis*, C: *S. epidermidis*).

No bacterial growth was observed in 2 of 5 different farms. From other farms, respectively; The factors of *L. garvieae* from farm 3, *L. garvieae*, and *B. Subtilis* from farm 4 and *S. epidermidis* from farm 5 were isolated.

3.2. Molecular identification and sequence analysis

DNAs obtained from isolated bacterial agents were used as templates in Real-Time PCR. Real-Time PCR procedure was performed to identify the isolates. Real-Time PCR results with Universal (27F-1492R) primers are given below (Figure 2).

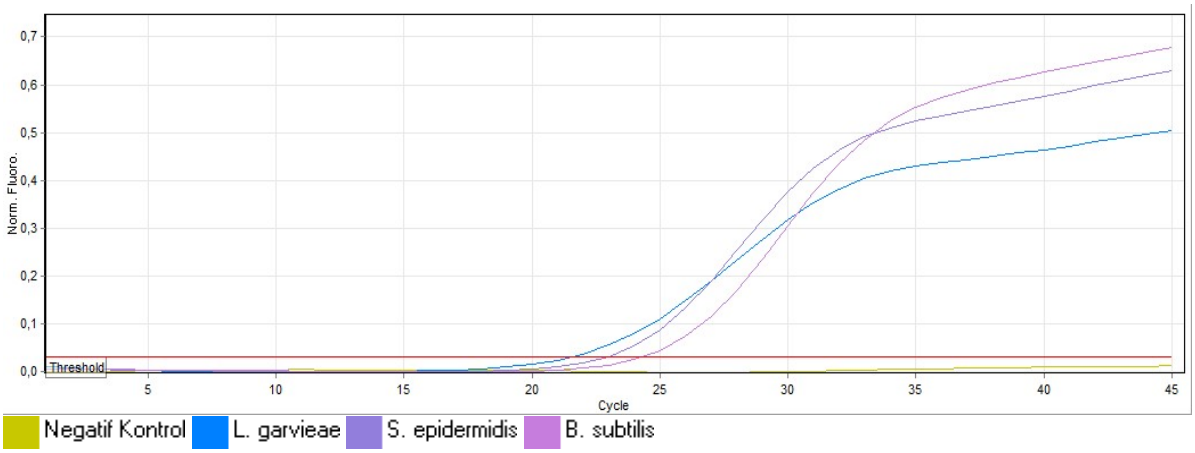

Figure 2. Real-Time PCR image performed with bacterial DNAs isolated in the study (Sigmoidal curves positive, negative control sample below the threshold value).

In line with Real-Time PCR results, it was observed that bacterial DNAs gave positive results in SYBRGreen-based fluorescent irradiation by binding with universal primers. According to the results of sequence analysis of Real-Time PCR amplicons obtained by Sanger One Way sequencing; Among the bacteria isolated in the study, *B. subtilis* were similar with 97.88%, *S. epidermidis* 98.67% and *L. garvieae* 90.77% (Figure 3).

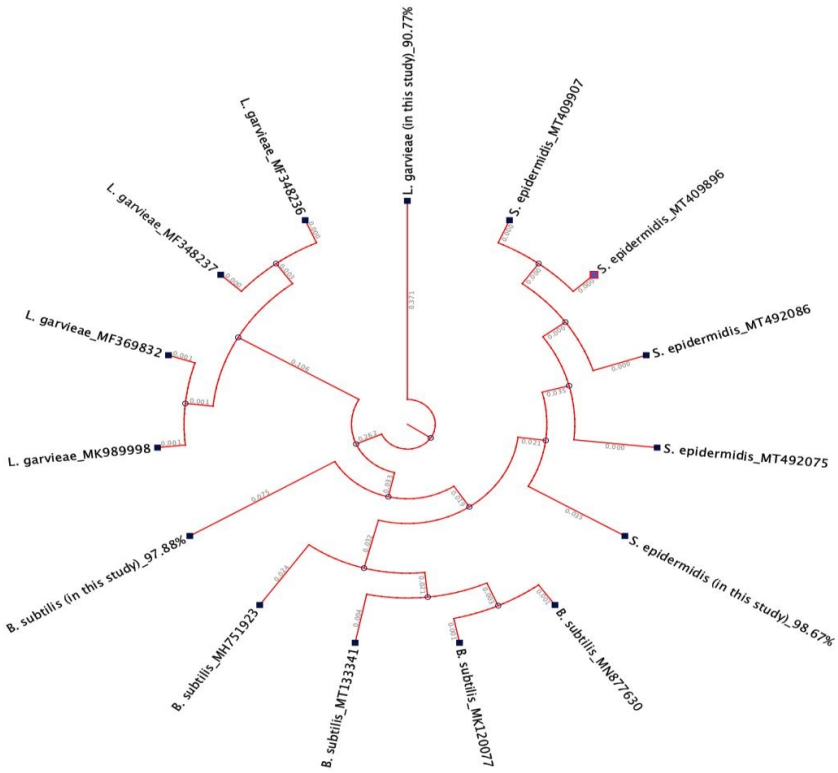

Figure 3. Sequence identifications of isolated bacteria and their closeness to the closest sequences.

These results also show similarity rates between the isolates in the same area isolated. As a result of the Blast process of the nucleic acid sequence performed on NCBI and CLC web bases, all of the isolated bacteria had 97-99% sequence overlap.

3.3. Enzyme activity results

When the graphic of brain tissue AChE levels change in infected fish was examined, the change of AChE levels among other infected fish groups of control group fish was found to be statistically significant (p<0.05). It was determined that the brain tissue AChE enzyme activity of infected fish decreased significantly compared to the control group fish (Fig. 4).

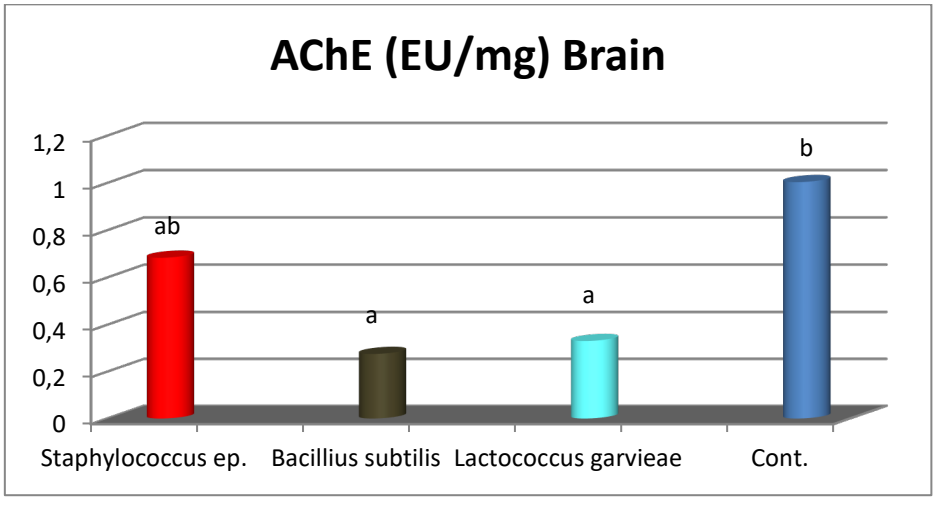

Figure 4. Change of brain tissue AChE levels in infected fish (p<0.05).

When the liver tissue AChE levels change of infected fish was examined, it was observed that all three bacterial species showed a significant decrease compared to the control group. It was determined that there was a significant decrease in the fish with *Staphylococcus epidermidis* bacteria (Figure 5).

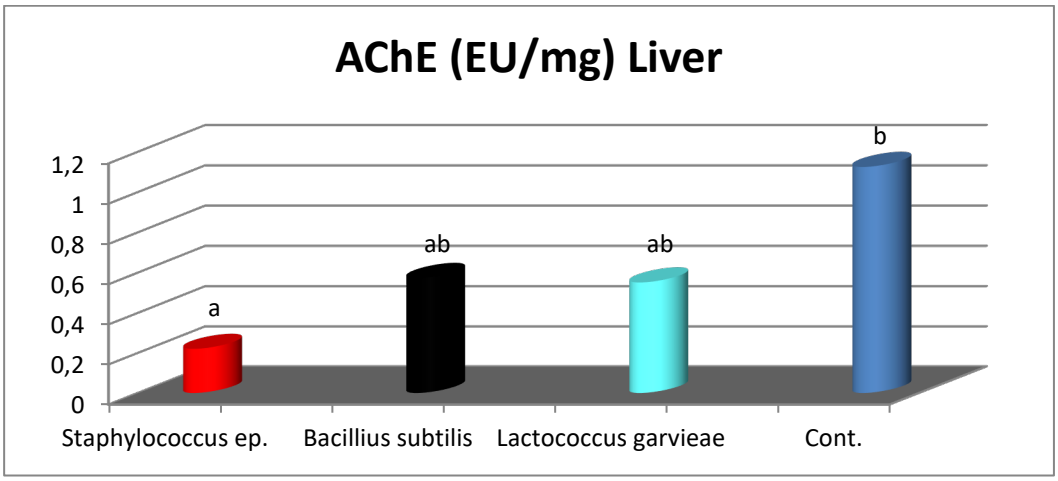

Figure 5. Change of liver tissue AChE levels in infected fish (p<0.05)**.**

The change in brain tissue BChE levels in all of three bacterial infection groups in fish showed a statistically significant change (p<0.005). It has been observed that the BChE level of trouts with *L. garvieae* bacteria decreased more than other groups (Figure 6).

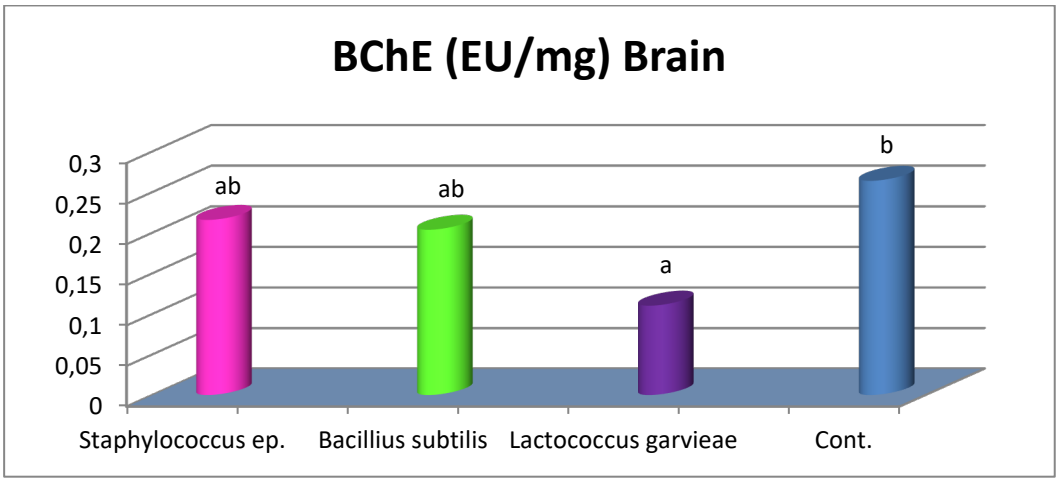

Figure 6. Change of brain tissue BChE levels in infected fish (p<0.05).

When the liver tissue BChE level change graph was examined, it was found that the enzyme levels of infected trout groups showed a statistically significant decrease. As in brain tissue, it was observed that the trout with *L. garvieae* bacteria was lower in BChE levels than in other groups (Fig. 7).

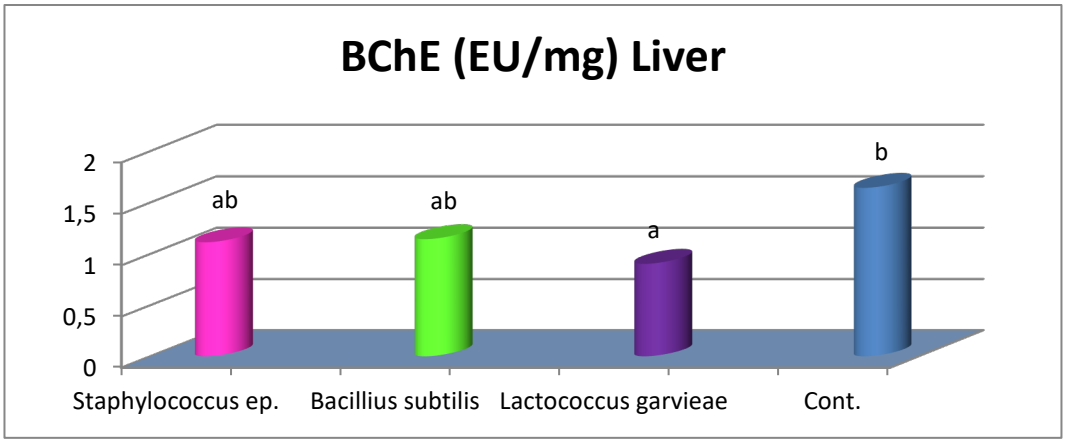

Figure 7. Change of liver tissue BChE levels of infected fish (p<0.05).

4. Discussion and Conclusion

Infections occurring in the aquaculture sector cause economic losses. It has been reported that bacterial pathogens are responsible for 54.9% of these infections, viruses for 22.6%, mycotic for 3.1%, and parasitic agents for 19.4% (Dhar et al., 2014). It is reported that *L. garvieae* is among the most important factors in bacterial studies carried out in the field of aquaculture. The isolation and identification period of the disease agents is essential for the ongoing diseases' treatment process (Balta and Dengiz Balta, 2019).

Nowadays, DNA-RNA isolations performed with automated robots can be performed in a much shorter time than manual studies. The fast and reliable isolation of DNA and RNA before PCR analysis is also essential for the accuracy. It has been reported by some researchers that the studies performed with automatic isolation robots create more reliable and pure isolations (Pinchi et al., 2013). In this study, the QIAcube DNA-RNA-Protein isolation robot was used in the isolation method. The purity degree of all of the isolated DNA was found between 1.8-2.0, and the subsequent PCR process was started. Today, the first methods that come to mind in terms of rapid diagnosis are among the molecular methods performed by Real-Time PCR. The PCR method is preferred by most researchers in that it provides fast results and is more reliable than other methods (Das et al., 2016). In this study, bacteria-specific universal primers were used. The same universal primers have been reported by many researchers carrying out bacterial studies (Nadkarni et al., 2002). After PCR analysis, identification of the bacteria isolated in the study was performed by sequence analysis. As a result of the study, *L. garvieae*, *B. subtilis*, and *S. epidermidis* agents were isolated. Thanks to the identification of the factors with the sequence, identification can be made among all the species available in the gene bank. Among the sequence methods, NGS is reported as the newest technology, but it is also known as the costliest technique. Among other methods, the sanger method is also reliable and is applied at more affordable price ranges (Al-Hebshi et al., 2015).

The increase in acetylcholine levels in the cholinergic system contributes to the neuromodulation in the neurological system, explaining the formation of the proposed synapses in the cerebral cortex and cerebellum (Schwertz et al., 2016). AChE and BChE changes of juvenile Silver catfish (*Rhamdia quelen*) fish infected with *A. hydrophila* were investigated. In the study, it was stated that the infection affects the cholinergic system and reduces AChE and BChE levels (Baldissera et al., 2016). A study has been conducted to evaluate whether the bovine infected with *L. monocytogenes* has altered the cholinesterase activity and to modulate its neurotransmission. In the study, it was determined that Acetylcholine level increased, Acetylcholine esterase level decreased. It has been stated that enzyme inhibition may cause this condition (Jaguezeski et al., 2018). Baldissera et al., (2018) found that neurological enzymes are reduced in the cerebral fluids of silver catfish infected with *Streptococcus agalactiae* associated with the central nervous system. It is seen that the results of our study are compatible with other studies in the literature.

AChE has anti-inflammatory effects. It is reported by Das (2007) that this reduction in AChE activity is a probable proinflammatory response of metabolism and may result from less AChE

hydrolysis to reduce tissue damage. Also, some fish species have been found to have no BChE enzyme (Chuiko, 2000). There are very few studies on the cholinergic system in infected fish. In this study, it was found that cholinergic system enzymes (AChE and BChE) started to decrease in the brain and liver tissues of rainbow trout infected with three different bacterial species (Staphylococcus spp., *B. subtilis* and *L. garvieae*). As a result; It can be thought that the cholinergic system enzymes of trout-infected trout have undergone denaturation or inhibition or reduced the enzyme secretions due to the anti-inflammatory properties of the cholinergic system.

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