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Investigation of the effect of a commercial probiotic-based product containing enzymes on rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

In this study, the aim was to test the effect of a candidate biotechnological product developed by a commercial company and containing different probiotic bacteria and enzymes on juvenile rainbow trout (*O. mykiss*) and water quality. The product's protective effect against fungal infections in fish eggs and the pathogen *Aeromonas hydrophila* was also investigated, as were its effects on the fish's intestinal tissue. Fifty fish were placed in aquariums in two experimental setups (A and B). Different doses of the product were added to the feed in Group A and water for the fish in Group B. Four different bath doses (d1-d4) of the product were applied to fish in Group B. At the end of the study, fish in Group A demonstrated better health, growth, feed conversion, and survival rates, as well as water quality, compared to the other groups. The product was found to be effective in improving the fish's intestinal health, reducing fungal infections in their eggs, and preventing infection by *A. hydrophila*. The data obtained from this study aims to contribute to the development of our country's aquaculture sector.

Keywords: Rainbow Trout, *O. mykiss*, Probiotic, Egg, *Aeromonas hydrophila*

Introduction

Rainbow trout (*O. mykiss*) farming has developed rapidly in Turkey in recent years and has become an important sector contributing to the national economy (Çakmak et al., 2024; TÜİK, 2024). Turkey is the largest trout producer in Europe, with an annual production of 75,567 tons (FAO, 2024). Bacterial diseases resulting from many adverse conditions such as excessive stocking density, inadequate water quality, stress, and inadequate nutrition, along with the development of antibiotic resistance in pathogens and feed costs, pose a risk to the sustainable development of the sector and the health of cultured fish (Lieke et al., 2020; Simon et al., 2021; Deekshit et al., 2022). Nowadays, preventive measures including new biotechnological applications (such as prebiotics, probiotics and enzymes) have become increasingly important in order to control fish diseases and to strengthen the non-specific defence mechanism of fish for protection against diseases (Süzer et al., 2008; Merrifield et al., 2010; Lieke et al., 2020; Simon et al., 2021; Deekshit et al., 2022; Amenyogbe et al., 2024).

Probiotics are live bacterial and yeast cultures, or biological products contained within these cultures, that are beneficial to the digestive system and health of fish (Balcazar et al., 2006; Vine et al., 2006; Amenyogbe et al., 2024). These products increase fish immunity against diseases, inhibit pathogenic bacteria, promote growth, and help regulate water quality (Balcazar et al., 2006; Vine et al., 2006; Kristiansen et al., 2011; Amenyogbe et al., 2024). Probiotic products are applied by adding them to the fish growing water, by adding them to the feed, and by adding them to both the water and feed. Researchers have reported that there are different steps in selecting the probiotics (alone or mixed) to be used in fish and shellfish and determining the optimal doses, but first, the pathogenicity test of the product should be performed under in vivo conditions (Balcazar et al., 2006; Vine et al., 2006; Feckaninova et al., 2017).

Studies on the use of probiotics against fish pathogens (such as bacteria and fungi) are gaining increasing importance. Studies have shown that Lactobacillus species, which have a probiotic effect, can produce substances that inhibit the growth of pathogens that cause Aeromonas infections in freshwater and marine fish, and are therefore important for fish health (Feckaninova et al., 2017; Redhwan et al., 2024). Studies are also underway to identify probiotic bacteria effective in preventing saprolegniasis, a fungal infection seen in cultured fish that primarily affects fish eggs (González-Palacios et al., 2018; Fregeneda-Grandes et al., 2023). The bacterial and fungal cultures found in probiotic mixtures generally have a positive impact on fish health by producing various minerals and trace elements, vitamins, and digestive enzymes such as amylase, lipase, and protease. For this reason, in recent years, commercial products containing enzymes obtained from these microorganisms have been widely used successfully in fish feed production (Assan et al., 2022; Amenyogbe et al., 2024; Asha et al., 2024).

This study will investigate the effects of an enzyme-containing probiotic product (codenamed Enzyfish), produced in the laboratories of a commercial biotechnology company, on in vivo cultured rainbow trout fry. In addition to determining the effective dose of this product, the product's effects on growth, feed conversion, disease resistance, and water quality of the cultured trout fry will be comprehensively investigated. The product's antifungal activity on eggs and its protective effect against the pathogen *Aeromonas hydrophila* were also investigated.

Materials and Methods

Experimental Fish and Eggs

The fry used in this study were purchased from a private trout farm while they were still in the fertilised egg stage. After 15-20 days, the observed fish eggs developed into larvae and began their first feeding stage. In our study, 1,200 fish samples, weighing an average of 0.14 g, were used. Additionally, approximately 400 egg-stage samples were used in our study on fungal infections.

Application of the Commercial Probiotic

The study aimed to determine the effective dosage of an enzyme-based product (Enzyfish), produced by a commercial company and containing various probiotic bacteria. Company officials stated that the product contains enzymes derived from various probiotic bacteria. Because it is a new commercial product, no further information about its ingredients has been provided. Therefore, the relevant studies were conducted on young trout during their initial feeding phase over 45 days. The product was applied to the fish feed and water at different rates in line with the company's recommendations.

Commercially available powdered trout feed was used to feed the young fish. Following the manufacturer's recommendations, 1 mL of the product liquid was taken per 1 kg of fish feed and diluted in 10 mL of sterile saline solution (0.8%). This solution was then sprayed onto the powdered feed using a spray bottle, and the product-enriched feeds were prepared.

In the process of adding the product to the water as a bath, the main stock was first prepared, and 100 mL of the product was diluted 36 times in water. Different dilutions were then made from this stock, and four different doses (d1-d4) were applied to the aquariums (Table 1). Juvenile fish in the first feeding phase were kept in these aquariums for 14 days using the long-term bathing method. No product was added to the water of the test fish during the remaining 30-day trial period.

Experimental Design

To determine the effect of the product on fish eggs, three experimental groups (y1, y2, y3) and one control group (y4) were prepared, each containing 100 eggs. Fish eggs were incubated in egg development baskets. The temperature of the water in the experimental groups was measured as 17 °C, pH: 7.2, and the oxygen amount was measured as 5-6 (mg/L).

Three different doses of the product (5, 10, and 15 µL/L) were applied to the eggs via 20 minute, 1 hour, and 24 hour baths. The number of dead white eggs and the rate of fungal growth on the eggs were examined during this approximately 5 day study. Eggs were not subjected to experimental infection.

The experimental setups in this study were divided into two groups: A and B. Fifty fish were placed in each aquarium in each experimental group, and the study was conducted in three replicates. The product was added to the food of all fish in aquariums in group A (A1-A5). While four different application doses (d1-d4) of the product were added to the aquarium water by the bath method for the fish in group B (B1-B5) aquariums, no product was added to the feed.

Similarly, four different doses (d1-d4) of the product were added to the aquarium water using the long-term bath method, except for aquarium A5 (Table 1). Four different application doses (d1-d4) of the product were added to the aquarium water of fish in Group B (B1-B5) aquariums using the bath method. In contrast, no product was added to the feed. During the 45-day study, the aquariums were cleaned daily, and the ambient temperature, oxygen level, pH, ammonia, and nitrate levels were measured.

Experimental Challenge

An experimental infection setup was established to determine the effectiveness of a commercial product against *Aeromonas hydrophila*, a pathogenic fish pathogen. Four experimental groups (h1-h4) were formed, each containing approximately 35 fish, and the experimental infection study was conducted over 1 month. The fish in aquariums h1, h2 and h3 were given a 0.01 mL volume of bacterial solution with a density of 1x 10^6 mL^{-1} by injection into the muscle. The individuals in the control tank (h4) were also treated with the bacteria in the same manner, but the disease was not treated. After the infection occurred, fish in group h1, which had previously been fed the product, were fed only normal food. Fish in group h2 received the product added to their feed, and fish in group h2 received a 1 hour bath with the product. At the end of the study, the effectiveness of the product in treating the disease was investigated by comparing the percentage of fish that died with the percentage of surviving individuals.

Table 1. Addition of the product to aquarium water in the bathing method

Dose	Aquarium volume	Product quantity	Application period
d1	30 L	5.550 mL	14 days
d2	30 L	55.5 mL	14 days
d3	30 L	55,5 mL	14 days
d4	30 L	275 mL	14 days

Growth Performance Measurement

Growth indicators such as weight gain (WG), specific growth rate (SGR), survival rate and feed conversion ratio (FCR) were calculated for all experimental fish in groups A and B at the beginning and end of the feeding period according to the equations specified by Korkut et al. (2007).

$$\text{Weight gain (WG)} = \frac{\text{Mean final weight gm} - \text{Mean initial weight gm}}{\text{t(days)}}$$

$$\text{Specific growth rate (SGR)} = \frac{\log W2 - \log w1 \ \text{x}\ 100}{T2 - T1}$$

$$\text{Feed conversion ratio FCR} = \frac{\text{Total amount of feed consumed (gm)}}{\text{Wet weight gain (gm)}}$$

$$\text{Survival rate} = \frac{\text{Number of fish harvested x 100}}{\text{Number of fish stocked}}$$

Bacteriological Inoculations of Water

Water samples were taken twice a week from all aquariums containing fish, and these samples were inoculated onto PCA (Plate Count Agar) medium, which was used for total bacterial counts. Petri dishes were incubated at 22°C for 24-48 hours. At the end of this period, the number of bacterial colonies (cfu) grown on the petri dish was recorded, and changes in the bacterial load in the water throughout the study were recorded (Huss, 1988).

Histological Examination

In this study, tissue samples were taken from intestinal sections of fish larvae fed regular trout feed (N) and feed supplemented with the product for histological analysis. The tissue samples were fixed in a 10% formaldehyde solution and subjected to routine tissue processing and procedures used in our Faculty's Histology Laboratory. Preparations were stained with H&E. These sections were examined under a light microscope. Changes in the structure of the villi in the intestinal tissue taken from 5 fish in each group were investigated. The mean values of the number and length of villi in the intestinal tissue were calculated (Kristiansen et al., 2011; El-Kady et al., 2022; Marco et al., 2023; Asha et al., 2024).

Results and Discussion

Preventing Fungal Infection

Application of various bath doses of a probiotic containing enzymes to eggs showed that, except for d1, d2, d3, and d4 doses, reduced fungal infection in eggs. Considering the mortality rate in eggs, it was determined that the best results were obtained with the d3 dose applied every other day or for a minute every three days. Similar to the findings of other researchers, the addition of probiotic-containing products to water during bathing has been shown to have a protective effect against fungal infections in fish eggs (González-Palacios et al., 2018; Fregeneda-Grandes et al., 2023; Amenyogbe et al., 2024).

Improved Growth Performance

After the trial was completed, the development of the fish we used as material was examined, and it was noted that the fish in Group A1 developed much faster than those in Group B, with increased transverse muscle thickness in the fish's body and a distinct colouration in the fins (Figure 1).

Figure 1. Appearance of the fish at the end of the experiment

In our research on the determination of the effective dose in the fish in the trial groups of the commercial product, the weight increase of the fish in group A, the specific growth rate (SGR), the feeding (FCR) and the survival rates were found to be better than those of the other group (Table 2).

As a result of our study, It has been observed that the chances of the fish in group A are still high and that individuals are more willing and healthier against eating. More successful results have been obtained on the growth of the fish with the addition of both water and food to the fish in group A. The data we obtain show that probiotics contribute to scientific data showing that they can be used commercially to accelerate growth, increase feed efficiency and improve fish production safety by using antibiotics in fish and shellfish breeding (Vine, 2006; El-Kady et al., 2022; Asha et al., 2024).

Water Quality and Effect on Bacterial Load

At the end of the trials, some of the values related to water quality in the aquariums remained constant (such as temperature, oxygen and pH), while some of them had changed. In the aquariums of the A and B groups, variability in ammonia and phosphate levels in water was observed, while the nitrate level was always measured low. However, when the total bacterial load in the water was examined, it was observed that there was a significant decrease at the end of the start and trial in all aquariums. In group B5, which was not added to the aquarium juice, the bacterial load in water increased at the end of the experiment. With this, it is noteworthy that algae in A4, A5 and B4 occurred in aquariums (Table 2).

When the effect of the product on water quality values in the A and B groups is examined, there are no major differences, as stated by the researchers, while measured in optimal values for fish (Sunithha & Krishna, 2016; al-Kady et al., 2022; Redhwan et al., 2024). In addition, when the bacterial load in the water in the essay aquariums was examined, the values in group B were higher than those in group A. The fact that the product added to water and eating is effective in reducing the bacterial load of water is consistent with the results of the previous studies (Sunithha & Krishna, 2016; Gamoori et al., 2022; Redhwan et al., 2024).

Development of Gut Tissue

The experimental findings of this study demonstrated that the probiotic bacteria present in our product contributed to the development of intestinal tissue in trout fry, as previously reported (Bagheri et al., 2008; Kristiansen et al., 2011; Asha et al., 2024).

Table 2. Findings of the experiments in groups A and B

	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5
Feed probiotic addition	X	X	X	X	X	-	-	-	-	-
Bathroom application	D1	D2	D3	D4	-	D1	D2	D3	D4	-
Survival rate (%)	94	86	85	86	88	86	85	84	83	79
FCR	0.8	1,04	1.2	1	0.9	1.13	1.33	1.2	1	1.4
Water quality values										
Water temperature (°C)	17-18	17.1-7.8	17.2-7.8	17.4	17-17.5	17.1	16.9-17.9	17-18	17-18	16.8-17
Oxygen in water (mg/L)	4.9-6.24	5.35-.22	4.8-6.44	5.06-5.91	5.34-6.24	5.72-6	4.9-6.2	4.77-6.11	5.4-6.1	4.72-5.7
pH	7.2-7.6	7.4	7.4	7.4-7.6	7.2-7.6	7.4	7.4	7.4-7.6	7.2-7.6	7.2-7.6
Ammonia (mg/L)	0.5-1	1-2.5	2-5	2.5-7	0.1-0.5	0.5-5	1-5	1-5	0,5-5	5-7
Phosphate (mg/L)	1-5	1-5	2-5	2-7	1-5	1-5	1-5	1-5	2-7	1-5
Nitrate (mg/L)	0	0	0	0	0	0	0	0	0	0
Salinity	0	0	1	3	0	0	0	1	3	0
Algae in water	-	-	-	+	+	-	-	-	+	-
Bacterial load in water (cfu/mL^{-1})	$2x10^6$ — $1x10^3$	$1.9x10^6$ — $2.1x10^4$	$1.2x10^6$ — $2x10^4$	$1.1x10^6$ — $1.6x10^4$	$2.8x10^6$ — $2x10^4$	$2.6x10^6$ — $2x10^4$	$1x10^6$ — $3,1x10^4$	$1.7x10^6$ — $5x10^4$	$3.7x10^6$ — $4 x10^4$	$3x10^6$ — $2.6x10^6$

Differences have been observed in the appearance of tissues obtained as a result of histological studies on the gut tissue of the trout cubs fed by regular feed (N) and commercial product added (F) (Figure 2). Intestinal samples from 5 fish in each group were examined. Approximately 18 villus were seen in the intestinal cross -section of the fish in the N group. At the same time, their length towards the lumen was approximately 100 µm, and it was measured as 100 µm in the thickness of the outer round muscle layer of the gut tissue. It is noteworthy that the villus numbers of the fish in the commercial product (F) have increased to 25, the villus length was generally more than 100 µm, and the outer muscle layer became thicker than the other group.

Researchers reported that different feed additives (enzymes, prebiotics, probiotics) contribute to the healthy development of fish larvae, causing changes in the villi in the intestines due to the increase in absorption ability (Kristiansen et al., 2022; El-Kady et al., 2024). In the histological study we have made, the increase in the number and length of the villi of the fish fed with the product enriched with the product is beneficial in the digestion of the enzymes in the product.

Increasing Fish Health

As a result of the experimental infection study, the disease occurred 2 days after the *A. hydrophila* bacteria were given to fish by injection method, and fish deaths started accordingly. Experimental groups and data obtained within the scope of the 30-day study are shown in Table 3. At the end of the study, the product had a preventive and therapeutic effect against experimental infection with *A. hydrophila*. As in the h1 group, the most effective application in the treatment of the disease was obtained as a result of the bath applications performed in fish fed before the disease (min. 15 days ago) and in the stage after the start of the disease. *A. hydrophila* is a bacterium that lives in freshwater fish and is found in the intestinal flora of the fish. The stress of the fish due to adverse environmental conditions causes the disease. Similar to other studies, in our experimental infection study, it has been shown that the product is effective in the prevention and treatment of bacterial diseases in celestial trout (Fečkaninová et al., 2017; Simon et al., 2021).

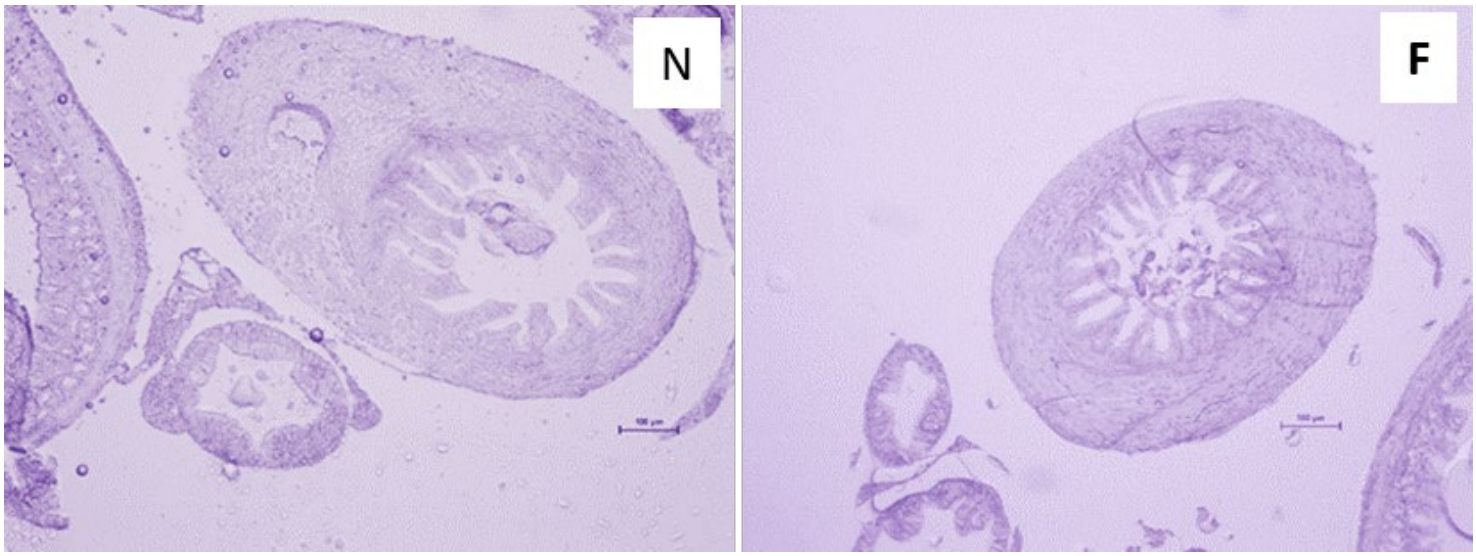

Figure 2. Appearance of intestinal sections of fish fed regular feed (N) and commercial product (F) (H&E)

Table 3. Findings on infected experimental groups with *A. hydrophila*

	h1	h2	h3	h4
Number of fish	35	35	35	35
The probiotic added before the disease feeding with feed	+	-	-	-
Eating probiotics after the development of the disease	-	+	-	-
Nutrition with normal feed after disease	+	-	+	+
Short-term bathroom application (2 times)	-	-	+	-
Number of dead fish	2	5	3	30
Ratio of survivors (%)	95	86	92	14

Conclusion

Probiotics are preferred, environmentally friendly products for increasing fish welfare and feed efficiency in sustainable aquaculture cultivation, and to protect fish health. It shows that commercial probiotics developed by adding enzymes, similar to today's biotechnological developments, are much more effective in increasing the health of fish, the immune system and production performance (Assan et al., 2022; Amenyogbe et al., 2024; Asha et al., 2024).

As a result of this study, the effects of adding the candidate biotechnological product (Enzyfish) considered for commercialisation or applying a bathing method to the water were tested on the growth and health of the fish. With the results obtained from this study, it was aimed to contribute to the development of the cultural fisheries sector in our country, as well as to shed light on the domestic studies to be carried out in the following years.

Compliance with Ethical Standards

Conflict of interest: The author declares no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The local ethics committee report was received at İstanbul University, Animal Experiment Local Ethic Committee Report at 2015/76 report number and 05.011.2015 report date.

Data availability: The data will be made available upon request from the author.

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Disclosure: -

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