Aquaculture International (2025) 33:208
https://doi.org/10.1007/s10499-025-01885-1

RESEARCH

Investigation of the probiotic effects of *Lactobacillus sakei* 2–3 and *Candida zeylanoides* Y12-3 strains in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792)

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Received: 25 December 2024 / Accepted: 13 February 2025 / Published online: 26 February 2025

Abstract
This study aimed to assess individual and combined effects of candidate probiotic strains *C. zeylanoides* Y12-3 and *L. sakei* 2–3 on growth, hematological parameters, serum immunological parameters, serum biochemistry, histopathology, histomorphology, expression of immune and antioxidant enzyme genes, and disease resistance against *L. garvieae* in rainbow trout. The fish were fed four different feeds (control, *L. sakei* 2–3, *C. zeylanoides* Y12-3, and *L. sakei* 2–3 + *C. zeylanoides* Y12-3) for 60 days. At the end of the experiment, growth parameters, serum glucose levels, serum lysozyme activity, and expression of immune and antioxidant enzyme genes were significantly increased in the probiotic groups. Additionally, triglyceride levels decreased in the probiotic groups compared to the control group, whereas serum ALT levels did not change. The villus width and the number of goblet cells increased in the proximal intestines of the fish in *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups. *L. sakei* 2–3 showed higher superoxide anion production, expression of immune genes (IgM, IL-B1, lysozyme, TNF-α, HSP70) in the kidney, antioxidant enzyme genes (GPX, GST, SOD) in the liver compared to *C. zeylanoides*. TNF-α, HSP70), and antioxidant enzyme genes (GPX, GST, SOD) compared to *C. zeylanoides*. *L. sakei* and the combination of *L. sakei* + *C. zeylanoides* provided resistance to *L. garvieae* compared to the control group. However, *C. zeylanoides* was similar to the other two probiotic groups regarding disease resistance against *L. garvieae*. However, histopathological examinations revealed reversible changes in the proximal intestine, anterior kidney, and liver of fish in the *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups. Hence, future studies are still required to explore the effects of shorter-term use of the *C. zeylanoides* strain in rainbow trout to prevent undesirable effects on tissues. In brief, the findings, as mentioned above, showed that *L. sakei* 2–3 and *C. zeylanoides* Y12-3 could be potential probiotic candidates for use in rainbow trout farming. Moreover, the probiotic effects of both strains on different fish species should also be studied.

Keywords Rainbow trout · Probiotic · Yeast · Lactic acid bacteria · Disease resistance · *L. garvieae*

Handling Editor: Daniel Merrifield

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Introduction

Aquaculture is crucial in providing a sustainable and reliable food source for the growing world population. Intensive aquaculture methods have been adopted to maintain high production levels, which has increased the risk of disease outbreaks due to the high density of aquaculture production and breeding. Many chemical compounds and antibiotics have been used to prevent and treat diseases. However, the use of these compounds has caused many problems, the most prominent of which is their indirect impact on human health and the development of antibiotic-resistant bacterial strains. Therefore, it has become important and urgent to explore environmentally friendly alternatives to protect health and enhance the growth of aquaculture (Dawood et al. 2019).

The use of probiotics in the feeds of cultured fish and shellfish is one of the most significant advancements for sustainable aquaculture, as they can promote growth and improve overall health (Dawood et al. 2019). Probiotics have also been shown to impact water quality positively (Hlordzi et al. 2020).

Probiotics are "live microorganisms that benefit the host when administered in adequate amounts" (Gatesoupe 1999). Probiotics influence weight gain in aquatic organisms, enhance health status, boost appetite, improve food palatability, and help break down indigestible food substances. They also produce vitamins, increase intestinal absorption, mitigate some toxin effects, reduce pathogen infections, compete for binding sites in the intestine, and promote the formation of peroxides, bacteriocins, lysozymes, and other physiological and immunological benefits. Probiotics regulate the development and selectivity of gut flora by minimizing the impact of harmful microorganisms. They support innate defense mechanisms against diseases by the production of inhibitory metabolites and enhance the tolerance of aquatic animals to stress caused by various environmental factors (Dawood et al 2019). With all these beneficial properties, bacterial or yeast-type probiotics are successfully used to solve many problems encountered in aquaculture (Borrelli et al. 2016; Vargas-Albores et al. 2021).

Lactic acid bacteria (LAB) are well known for their lack of virulence factors, ability to compete with pathogenic bacteria by producing antimicrobial inhibitors, and capacity to reproduce and spread throughout the digestive tract. They compete with pathogens for attachment on the intestinal wall, stimulate the non-specific immune response, and enhance the production of enzymes (such as amylase, lipase, and protease) within the intestine. These microorganisms are used as probiotics in aquaculture due to their ability to alter pH and redox potential, exhibit antagonistic effects on pathogen development through the production of hydrogen peroxide and bacteriocins, and neutralize pathogenic bacterial toxins and metabolites (Farzanfar 2006; Mota et al. 2006; Gatesoupe 2008; Askarian et al. 2011). The positive effect on fish growth is believed to result from the increased secretion of exoenzymes produced by lactic acid bacteria, particularly those from the Lactobacillus genus (Moriarty 1996; 1998; Askarian et al. 2011).

Yeasts are the second group of microorganisms, after bacteria, with potential applications in aquaculture (Caruffo et al. 2015; Banu et al. 2020). *S. cerevisiae* has been widely used as a probiotic in cultured fish species because it improves growth performance and immune responses and reduces stress (Li and Gatlin 2003; Abdel-Tawwab et al. 2008; Hoseinifar et al. 2011; Banu et al. 2020). The probiotic effect of *Debaryomyces hansenii* isolated from fish has also been investigated in sea bream and leopard grouper (Reyes-Becerril et al. 2008, 2011; Hernández-Contreras et al. 2021). In addition, different yeast species isolated from fish have been studied for their probiotic effects in

aquaculture: *Yarrowia lipolytica* 242, *Candida deformans* 154, *Cryptococcus laurentii* 21, *Metschnikowia viticola* 15, and *Rhodotorula mucilaginosa* 9–1 in zebrafish (*Danio rerio*) (Caruffo et al. 2015), *Yarrowia lipolytica* N-6 in Pacific red snapper (Alamillo et al. 2017), *Hyphopichia pseudoburtonii* 12–1 and *Candida zeylanoides* 12–3 in rainbow trout (Çaylı Bektaş et al. 2024).

Lactococcus garvieae is a gram-positive bacterium known to induce high mortality rates in fish. It can lead to hemorrhagic septicemia and meningoencephalitis in various fish species, resulting in significant economic losses in aquaculture (Pérez-Sánchez et al. 2011; Vendrell et al. 2006). Since 2001, outbreaks of lactococcosis have been frequently reported in rainbow trout in Turkey (Diler et al. 2002; Duman et al. 2020; Sezginet al. 2023). Recent studies have shown that a new pathogen, *Lactococcus petauri*, causes lactococcosis infections similar to those caused by *L. garvieae* (Altinok et al. 2022; Saticioglu et al. 2023). Although antibiotics are used in attempts to treat lactococcosis outbreaks in aquaculture, these treatments are often ineffective, and the indiscriminate use of antibiotics leads to resistance (Pérez-Sánchez et al. 2011; Romalde and Toranzo 2002; Vendrell et al. 2006).

It has been reported that lactic acid bacterial strains, including *Lactobacillus mesenteroides* CLFP 196, *Lactobacillus plantarum* strains (Vendrell et al. 2008), *L. cremoris* (Araújo et al. 2015), *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus sakei* (Didinen et al. 2018), as well as *Enterococcus faecalis* UGRA10 (Baños et al. 2019), provide disease resistance against lactococcosis caused by *L. garvieae* in rainbow trout. Additionally, yeast strains such as *Hyphopichia pseudoburtonii* Y12-1 (10^5 cfu/g feed) and *Candida zeylanoides* Y12-3 (10^5 cfu/g feed) have also been shown to confer resistance to lactococcosis caused by *L. garvieae* in rainbow trout.

This study aimed to evaluate the effects of dietary administration of potential probiotic *C. zeylanoides* Y12 and *L. sakei* 2–3 strains, individually and in combination, on growth parameters, hematological parameters, serum immunological parameters, serum biochemistry, histopathological and histomorphological changes in tissues, expression of immunity-related genes in the kidney, antioxidant enzyme activities in liver, and disease resistance against *L. garvieae* in rainbow trout.

Materials and methods

Diet preparation

Candidate probiotic *L. sakei* 2–3 (Didinen et al.2018) and *C. zeylanoides* Y12-3 (Çaylı Bektas et al. 2024), used in the trial feeds were isolated from rainbow trout in our previous studies and stored in a deep freezer at − 80 °C.

De Man Rogosa Sharpe (MRS) broth (Biolife) was used to culture lactic acid bacterial strain, *L. sakei* 2–3, to be added to the experiment feeds. *L. sakei* 2–3 strains were inoculated into 500 mL MRS and incubated at 25 °C for 24 h. The plate counting method with MRS agar counted the lactic acid bacteria. After incubation, the 24-h culture was centrifuged at 5000 rpm at 4 °C for 15 min, and the supernatant was discarded. To obtain the target *L. sakei* 2–3 concentration (10^8 cfu/g) in the feed, bacterial pellets from two 500 mL *L. sakei* cultures were harvested and incorporated into 1 kg of feed.

For the culturing of *C. zeylanoides* Y12-3 yeast strain, Yeast-Peptone-Dextrose (YPD) broth (Condalab) and Yeast Mold (YM) Broth (Difco) broth in large volumes (500 mL) did not provide adequate yeast density. Therefore, Yeast Extract Glucose Chloramphenicol

(YGC) Agar (Biolife) was used for *C. zeylanoides* Y12-3 culture. To reach the target *C. zeylanoides* (10^8 cfu/g) in the feed, *C. zeylanoides* was spread across the surface of 14 Petri dishes containing YGC agar and incubated at 25 °C for 48 h. After incubation, yeast cells suspended in PBS (approximately 10 mL) from each Petri dish were collected into sterile bottles using an automatic pipette. The yeast cells were counted using the plate counting method with YGC agar. Yeast cells in PBS (approximately 150 mL) were then harvested by centrifugation at 5000 rpm for 15 min in a refrigerated centrifuge at + 4 °C and were added to 1 kg of feed.

The harvested *C. zeylanoides* Y12-3 and *L. sakei* 2–3 pellets were mixed with anchovy oil and added to extruded commercial un-oiled trout feed (HEMAKS, Gaziantep, Türkiye). The feed preparation process followed the procedure described in the literature (Yılmaz and Ergün, 2018). Briefly, 880 g of feed was preheated at 40 °C for 1.5–2 h, and then 120 g of anchovy fish oil was slowly using a drum mixer. The experimental groups are as follows:

1. For the control group, 120 g of fish oil was added to 880 g of feed.
2. For the *L. sakei* group, 120 g of fish oil and *L. sakei* 2–3 strain (10^8 cfu/g) (Didinen et al. 2018) were added to 880 g of feed.
3. For the *C. zeylanoides* group, 120 g of oil and *C. zeylanoides* Y12-3 strain (10^8 cfu/g) (Liao et al. 2022) were added to 880 g of feed.
4. For the *L. sakei* + *C. zeylanoides* group, 120 g fish oil, *L. sakei* 2–3 (10^8 cfu/g) and *C. zeylanoides* Y12-3 (10^8 cfu/g) were added to 880 g of feed.

The final protein/lipid ratio was approximately 46%/18% in trial feeds. Before the feeding experiment, it was determined how long candidate probiotics maintained their numbers in the feed when stored at + 4 °C. For this purpose, yeast in YGC agar and lactic acid bacteria MRS Agar were counted on the 1st, 7th, 15th, and 21st days in the trial feeds stored at + 4 °C. This helped establish the frequency with which the feeds needed to be prepared during the experiment.

Table 1 presents the numbers of yeast and lactic acid bacteria (cfu/g) in the trial feeds. In the *L. sakei* group, the bacterial count decreased on the 21st day. As a result, trial feeds were prepared every 15 days.

Table 1 Lactic acid bacteria (LAB) and yeast counts (cfu/g) in the experiment feeds

Experiment feed		1st day	7th day	15th day	21st day
Control	LAB	$<10^1$	$<10^1$	$<10^1$	$<10^1$
	Yeast	$<10^1$	$<10^1$	$<10^1$	$<10^1$
L. sakei	LAB	1.4×10^8	1.1×10^8	1×10^8	7.6×10^6
	Yeast	$<10^1$	$<10^1$	$<10^1$	$<10^1$
C. zeylanoides	LAB	$<10^1$	$<10^1$	$<10^1$	$<10^1$
	Yeast	3×10^8	1.7×10^8	1.2×10^8	1.26×10^8
L. sakei + *C. zeylanoides*	LAB	3×10^8	2.41×10^8	1.3×10^8	1×10^8
	Yeast	1.3×10^8	1.14×10^8	1.1×10^8	1.69×10^8

Feeding experiment

The rainbow trout (33.01 ± 0.04 g) used in the experiment were obtained from the Keskin Trout facility in the Bayramiç district of Çanakkale province. The fish were acclimated for 2 weeks before the experiment began. During this period, the fish were fed with commercial trout pellet (size: 3 mm, protein: 46%, lipid: 18%, HEMAKS, Gaziantep, Türkiye) food twice daily until satiation. Before the start of the experiment, each fish was externally examined following the EPA guidelines to evaluate its health condition qualitatively (Klemm et al. 1993). The examination confirmed that the fish were in good health. At the end of the acclimation period, 30 fish were randomly assigned to each experiment tank with similar lengths and weights. The experiment consisted of 4 groups, with 3 repetitions, and 360 fish (4 groups × 3 repetitions × 30 fish per tank) were used. The water flow rate was set at approximately 160 L/h.

During the experiment, the fish fed four different feeds (control, *L. sakei* 2–3 (10^8 cfu/g), *C. zeylanoides* Y12-3 (10^8 cfu/g), and a combination of *L. sakei* 2–3 (10^8 cfu/g) + *C. zeylanoides* Y12-3 (10^8 cfu/g) at 2% of their live weight twice a day for 60 days.

Physical and chemical water quality analysis

During the experiment, temperature, oxygen, conductivity, and pH values in the water were measured with a water parameter device (AZ 86031). Total ammonia, nitrite, and nitrate levels were measured with an Optizen POP UV/VIS spectrophotometer using a commercial kit (Spectroquant®).

Growth performance, feed conversion calculation

The following formulas were used to calculate growth performance and feed conversion:

$$Weight\ gain\ (\%) = \frac{(Final\ weight\ g - Initial\ weight\ g)}{Initial\ weight\ g} \times 100$$

$$Specific\ growth\ rate\ (SGR)\left(\%d^{-1}\right) = \frac{In\ (Final\ weight\ g) - In\ (Initial\ weight\ g)}{experiment\ period\ days} \times 100$$

$$Feed\ conversion\ ratio\ (FCR)(\%) = \frac{Weight\ gain(g)}{Feed\ consumption\ (g)} \times 100$$

Enumeration of LAB and yeast in intestines

After the feeding trial, three fish from each group were euthanized using clove oil (200 mg/L), and the posterior part of their intestines was collected. The samples were then dissected and serially diluted with PBS. LAB and yeast were enumerated on MRS and YGC agar using the plate counting method, respectively.

Blood sampling and analysis

At the end of the experiment, three fish were randomly selected from each tank (9 fish/group) for blood analysis. The fish were anesthetized with clove oil (20 mg/L) (Iversen et al. 2003). After thoroughly cleaning the posterior part of the anal fin with alcohol to prevent mucosa contamination, blood was collected as quickly as possible by inserting a 2.5 mL plastic syringe into the caudal vein, ensuring minimal harm to the fish. Blood samples were transferred to tubes containing K_2EDTA and gel serum tubes. The samples in K_2EDTA tubes were used for hematological measurements, superoxide anion production, and respiratory burst activity (NBT test). Blood samples collected in gel serum tubes were centrifuged at 5000 rpm for 5 min. The serum obtained was then stored at − 80 °C for biochemical analysis and lysozyme and MPO activities (Yılmaz and Ergun, 2018).

Hematological assays

Hematological parameters, including red blood cell count (RBC), hemoglobin concentration (Hgb), and hematocrit level (Hct), were measured using an automated blood counter (Mindray/BC 3000 Plus), which had previously been used for trout in fish studies at the laboratory of Çanakkale Onsekiz Mart University's Faculty of Marine Sciences and Technology. The results from this device were compared to traditional counting and analysis methods in earlier studies (Yılmaz and Ergün, 2018; Bayïzït et al. 2023), which showed no significant statistical differences. Therefore, the same device was used for hematological analysis in this study. It is important to note that this device is suitable only for RBC, Hgb, and Hct evaluations.

Immunological analysis

Superoxide anion production (OD at 620 nm), respiratory burst activity of neutrophils and monocytes (OD at 620 nm), serum lysozyme activity (µg/mL), and myeloperoxidase activity (OD at 450 nm) were evaluated according to our previously published paper (Yılmaz and Ergün, 2018).

Biochemical analysis

Biochemical analyses were performed on the serum using a spectrophotometer using the Bioanalytic kit (Bioanalytic Diagnostic Industry, Co). In the experiment, the following biochemical parameters were determined: glucose, albumin, globulin, total protein, triglyceride, cholesterol (KOL), aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP).

Experimental infection with *L. garvieae*

Determination of LD50 value of *L. garvieae*

In a previous study, the LD50 dose of the *Lactococcus garvieae* SY-LG1 (GenBank Access Number: KY118086) strain, isolated from sick rainbow trout.

The *L. garvieae* SY-LG1, which had previously achieved the desired mortality rate in earlier research, was used in this study. However, the LD50 dose was recalculated to account for any potential changes in the LD50 value of the *L. garvieae* SY-LG1 strain in the fish to be used. For this purpose, *L. garvieae* SY-LG1 was injected into 20 fish at five different doses (1×10^5 cfu/fish, 1×10^6 cfu/fish, 1×10^7 cfu/fish, 1×10^8 cfu/fish, and 1×10^9 cfu/fish). Fish mortality was recorded over 20 days (Chang et al. 2014). *L. garvieae* SY-LG1 was reisolated from the kidney, liver, and spleen of freshly dead fish at 24 °C for 48 h (Yilmaz et al. 2024).

Challenge experiment

After the feeding experiment, 20 fish (60 fish/group) from each tank were anesthetized with 20 mg/L clove oil (Iversen et al. 2003). IP injected these fish with *L. garvieae* SY-LG1 at the LD50 dose (1×10^8 cfu/fish). Dead fish were collected, and mortality was recorded daily for 30 days. The survival rates (%), the mortality rate (%), and relative percent survival (RPS) were calculated.

$$Relative\ percentage\ survival\ (RPS\%) = 1 - \frac{(Mortality)(\%)in\ treated\ group}{(Mortality(\%)in\ control\ group)} \times 100$$

Histopathological analysis

In this study, the proximal intestine, liver, and anterior kidney tissues of the fish were examined histologically. The proximal part of the intestine, starting from the end of the stomach, was used for intestinal histology. The feces in the proximal intestine were carefully cleaned using physiological saline without damaging the tissue. The intestines were sectioned into 1 cm cross-sections. Liver tissues were removed immediately after dissection and cut into smaller pieces using a scalpel. For kidney tissue, the anterior kidneys were collected. All tissue samples were fixed in Bouin's solution for 8 h, after which they were stored in 70% ethyl alcohol at room temperature, away from light, until preparation. The tissue samples were passed through 80%, 85%, 90%, and 96% alcohol, followed by absolute alcohol, xylene, and a 1:1 xylene-paraffin, before being embedded in paraffin. Paraffin blocks were sectioned to a thickness of 5 µm using a Leica rotary microtome. Three different slides were prepared from each tissue sample. Sections were stained with Hematoxylin & Eosin for histological and histomorphological examinations. All slides were examined under an Olympus CX21 light microscope. Histomorphological measurements were taken, and photographs were captured using the Olympus Analysis LS software and a camera adapted to an Olympus BX51 light microscope (Mills 2006).

Histomorphological measurements

Intestinal histomorphological measurements

In the cross-sections obtained from the proximal intestine, three different histological slides were used for histomorphological measurements of villus length and width (VL-VW) for each sample. For this purpose, each slide's villi of five different sizes (short, medium, and

long) were measured. The VL measurements measured the distance from the muscularis mucosa layer at the base of the villus to its apical part (including the microvilli). The VW measurements recorded the width of the median part of the villus. The total number of goblet cells per villus was also counted.

Liver histomorphological measurements

The diameters of hepatocytes were measured in liver sections. For this, 10 randomly selected hepatocytes from each liver section were evaluated. Three different slides were used for each group.

Gene expression analysis

Sampling

At the end of the 60-day feeding experiment, tissue samples were collected from the 9 fish per group (3 fish per tank). Kidney and liver samples from the rainbow trout were transferred to Eppendorf tubes, and RNA later solution (Sigma-Aldrich, lot no. #R0901) was added for sampling. The samples in the RNA later solution were kept at 4 °C overnight and then stored at − 20 °C until the day of gene expression analysis (McCarthy et al. 2013).

RNA extraction and cDNA synthesis

According to the manufacturer's instructions, total RNA was extracted from the samples using GeneMatrixTM extraction Kits (Cat. NO. E3598, Poland). The quality and purity of all RNA samples were assessed using a Multiskan GO (Thermo Fisher Scientific™) instrument at wavelengths of 260 and 280 nm. After the measurements, RNA samples were reverse transcribed into cDNA using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit, and the resulting samples were stored at − 20 °C.

Primers used in gene expression analysis

Primer sequences and NCBI GenBank accession numbers of target genes are provided in Table 2. Gene expressions of lysozyme, IL-1β, IL-10, HSP70, TNF-α, and IgM (heavy chain) were assessed in the anterior kidney tissues of fish, while gene expressions of Sod1, cat, gpx, and gst were evaluated in the liver tissues.

Thermal cycling, RT-qPCR, and gene expression analysis

Gene expression levels of the genes mentioned above were determined using the Applied Biosystems™ StepOne™ Real-Time PCR System (Fisher Scientific, USA). Beta-actin

Table 2 Primers used in gene expression analysis

Gene	Primer (5′−3′)	References
β-Actin	F5′ GGACTTTGAGCAGGAGATGG 3′ R5′ ATGATGGAGTTGTAGGTGGTCT 3′	Awad et al. (2011); Yılmaz and Ergün (2018)
IL-1β	F5′ ACCGAGTTCAAGGACAAGGA 3′ R5′ CATTCATCAGGACCCAGCAC 3′	Awad et al. (2011); Yılmaz and Ergün (2018)
IL-10	F5′ CGACTTTAAATCTCCCATCGAC 3′ R5′ GCATTGGACGATCTCTTTCTTC 3′	Gen no: AB118099
TNF-α	F5′ TCTTACCGCTGACACAGTGC 3′ R5′ AGAAGCCTGGCTGTAAACGA 3′	Evenhuis and Cleveland (2012); Yılmaz and Ergün (2018)
HSP70	F5′ CTGCTGCTGCTGGATGTG 3′ R5′ GCTGGTTGTCGGAGTAAGTG 3′	Gen no: AB062281.1
Lysozyme	F: 5′ ACAGCCGCTACTGGTGTGACG 3′ R: 5′ GCTGCTGCCGCACATAGAC 3′	Yar Ahmadi et al. (2014)
IgM-(heavy chain)	F: 5′ CAAACCGGTGGAAGCTACAT 3′ R: 5′ AGACGGCTGCTGCAGATATT 3′	Evenhuis and Cleveland (2012); Yılmaz and Ergün (2018)
Sod1	F: 5′ TGGTCCTGTGAAGCTGATTG 3′ R: 5′ TTGTCAGCTCCTGCAGTCAC 3′	Wischhusen et al. (2019) Gen no: AF469663.1
cat (catalase)	F: 5′ TGATGTCACACAGGTGCGTA 3′ R: 5′ GTGGGCTCAGTGTTGTTGAG 3′	Wischhusen et al. (2019) Gen no: BX087110.3
Gpx	F: 5′ CGAGCTCCATGAACGGTACG 3′ R: 5′ TGCTTCCCGTTCACATCCAC 3′	Fontagné et al. (2008)
Gst	F: 5′ TCGCTGACTGGACGAAAGGA 3′ R: 5′ CGAAGGTCCTCAACGCCATC 3′	Gen no: BX302932.3

(*β-actin*) was used as the housekeeping gene for normalization. RT-qPCR analysis was performed in a final volume of 20 µL using 0.5 µL of forward and reverse primers and a 5×HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia) kit. The thermocycling conditions steps for RT-qPCR were as follows: initial denaturation (12 min at 95 °C), followed by 15 s at 95 °C, then annealing and extension (60 s at 60 °C). Data analysis was performed using the 7500 System SDS software version 1.3.1 (Applied Biosystems). Gene expression levels were calculated using the Livak and Schmittgen ($2^{-\Delta\Delta Ct}$ method) (Livak and Schmittgen 2001).

Statistical analyses

Homogeneity and normality tests were conducted initially to assess the difference between the growth parameters, immunological parameters, serum biochemical parameters, hematological parameters, histomorphological measurements, immune-related gene expression, and antioxidant activity-related gene expression across groups after the feeding experiment. Non-homogeneous data were analyzed using the Tamhane test, irrespective of normality. The Tukey test was applied for data that exhibited normal and homogeneous distribution. The significance level was set at $p < 0.05$.

Kaplan–Meier analysis was performed to compare mortality rates following experimental infection, and pairwise comparisons were made using the log-rank (Mantel-Cox) test to identify differences between groups. The analysis was conducted using SPSS 19.0 (SPSS Statistics), with a significance level set at $p < 0.05$.

Results

Physical and chemical water quality findings

During the experiment, the following water parameters were recorded: temperature ranged from 14.1 to 16.2 °C, oxygen levels were between 6.9 and 7.8 mg/L, conductivity ranged from 405 to 455 µS/cm, pH values were between 7.0 and 7.6, total ammonia levels ranged from 0.011 to 0.015 mg/L, nitrite levels varied from 0.01 to 0.06 mg/L, and nitrate levels ranged from 0.19 to 0.25 mg/L.

Table 3 Growth parameters and feed conversion rates of rainbow trout in the control and probiotic groups

	Initial weight(g)	Final weight(g)	weight gain (%)	SGR (% Day^{-1})	FCR
Control	34.20 ± 0.01	58.19 ± 0.12^{c}	70.14 ± 0.33^{c}	0.88 ± 0.00^{c}	1.18 ± 0.00^{c}
L. sakei	34.15 ± 0.02	66.48 ± 0.41^{ab}	94.63 ± 1.21^{ab}	1.11 ± 0.01^{ab}	0.96 ± 0.01^{ab}
C. zeylanoides	34.15 ± 0.03	62.88 ± 0.04^{b}	84.09 ± 0.13^{b}	1.02 ± 0.00^{b}	0.99 ± 0.00^{b}
L. sakei + *C.zeylanoides*	34.17 ± 0.00	66.74 ± 0.04^{a}	95.35 ± 0.14^{a}	1.11 ± 0.00^{a}	0.96 ± 0.00^{a}

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 4 Lactic acid bacteria (LAB) and yeast counts (cfu/g) in intestines in the control and probiotic groups

Groups	Lactic acid bacteria	Yeast
Control	2.44 ± 0.05^{d}	4.19 ± 0.17^{c}
L. sakei	7.02 ± 0.04^{a}	3.35 ± 0.35^{d}
C. zeylanoides	2.88 ± 0.02^{c}	5.81 ± 0.17^{a}
L. sakei + *C. zeylanoides*	3.65 ± 0.12^{b}	5.22 ± 0.11^{b}

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 5 Immunological parameters of rainbow trout in control and probiotic groups

Groups	Lysozyme (µg/mL)	Myeloperoxidase (OD, 450 nm)	Respiratory burst (OD, 620 nm)	Superoxide anion (OD, 620 nm)
Control	8.28 ± 0.36^{c}	0.31 ± 0.02	0.22 ± 0.04^{b}	0.35 ± 0.04^{b}
L. sakei	14.78 ± 1.39^{b}	0.30 ± 0.01	0.29 ± 0.04^{a}	0.42 ± 0.04^{a}
C. zeylanoides	15.31 ± 0.85^{b}	0.31 ± 0.02	0.23 ± 0.05^{ab}	0.36 ± 0.05^{b}
L. sakei + *C. zeylanoides*	20.71 ± 1.79^{a}	0.36 ± 0.04	0.19 ± 0.03^{b}	0.32 ± 0.03^{ab}

Growth parameters and feed conversion rates

The growth parameters observed in the groups as a result of the feeding experiment are presented in Table 3. At the end of the experiment, the probiotic groups exhibited significant differences ($p < 0.05$) in final weights, body weight gains (%), and specific growth rates when compared to the control group. Additionally, the feed conversion rates (FCR) in the probiotic groups were significantly lower than the control group ($p < 0.05$) (Table 3).

LAB and yeast counts in ıntestines

At the end of the experiment, the counts of LAB and yeast in the intestines of the fish from each group are presented in Table 4.

Serum immune parameters

After the feeding experiment, serum lysozyme activity in fish in the probiotic groups was higher than in the control group ($p < 0.05$). None of the probiotic applications affected the myeloperoxidase value ($p > 0.05$). Respiratory burst activity and superoxide anion production were higher in the *L. sakei* group than in the control group ($p < 0.05$) (Table 5).

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 6 Biochemical parameters of rainbow trout in control and probiotic groups

	Control	*L. sakei*	*C. zeylanoides*	*L. sakei* + *C. zeylanoides*
Total protein (g/dL)	11.66 ± 0.27^{b}	14.73 ± 0.83^{a}	12.33 ± 0.32^{ab}	10.84 ± 0.86^{b}
Albumin (g/dL)	0.42 ± 0.01^{bc}	0.50 ± 0.03^{c}	0.36 ± 0.01^{ab}	0.30 ± 0.02^{a}
Globulin (g/dL)	11.24 ± 0.27^{b}	14.23 ± 0.80^{a}	11.96 ± 0.32^{ab}	10.53 ± 0.84^{b}
Glucose (mg/dL)	77.12 ± 3.73^{c}	105.98 ± 6.73^{a}	108.42 ± 2.81^{a}	98.57 ± 4.65^{b}
Triglyceride (mg/dL)	158.61 ± 15.32^{a}	102.99 ± 3.04^{b}	111.81 ± 4.69^{b}	91.56 ± 10.74^{b}
Cholesterol (mg/dL)	319.32 ± 23.73^{a}	297.17 ± 16.29^{b}	278.26 ± 12.64^{b}	237.19 ± 9.18^{b}
AST (U/L)	115.95 ± 4.38^{ab}	127.79 ± 8.11^{a}	104.33 ± 4.47^{bc}	87.82 ± 5.63^{c}
ALT (U/L)	13.30 ± 0.83	13.03 ± 0.30	11.49 ± 0.99	12.79 ± 0.50
LDH (U/L)	1390.65 ± 11.49	1321.11 ± 87.88	1042.90 ± 99.54	1098.49 ± 55.16
ALP (g/dL)	510.04 ± 24.47^{a}	408.43 ± 36.59^{ab}	429.26 ± 42.51^{ab}	345.05 ± 57.33^{b}

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Biochemical parameters

The serum biochemical parameters of the fish at the end of the feeding experiment are shown in Table 6. Serum glucose levels were significantly higher in all probiotic groups compared to the control group ($p < 0.05$). Total protein and globulin values in the *L. sakei* group were higher than those in the control group ($p < 0.05$). Probiotic treatments did not affect serum cholesterol levels ($p > 0.05$). The AST level in the *L. sakei* + *C. zeylanoides* group was lower compared to the control group. ALT levels were similar between the probiotic and control groups ($p > 0.05$). Serum triglyceride levels were lower in the probiotic groups compared to the control group ($p < 0.05$). No significant differences were observed in LDH and ALP levels across all groups ($p > 0.05$).

Hematological parameters

No alterations in hematological parameters were observed in any of the probiotic groups after the experiment ($p > 0.05$) (Table 7).

Table 7 Hematological parameters of rainbow trout in control and probiotic groups

	RBC (10^6 mm^{-3})	HGB (g/dL)	HCT (%)
Control	1.62 ± 0.04	8.99 ± 0.41	32.91 ± 0.74
L. sakei	1.63 ± 0.04	9.04 ± 0.24	33.95 ± 0.58
C. zeylanoides	1.58 ± 0.07	8.88 ± 0.43	33.52 ± 1.19
L. sakei + *C. zeylanoides*	1.50 ± 0.04	8.16 ± 0.28	31.95 ± 0.59

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 8 Survival rate (%), mortality rate (%), and relative percent survival (RPS) in control and probiotic groups

	Number of fish	Mortality (%)	Survival rate (%)	RPS
Control	60	71.7^{b}	28.3^{b}	-
L. sakei	60	40.0^{a}	60.0^{a}	44.21
C. zeylanoides	60	51.7^{ab}	48.3^{ab}	27.89
L. sakei + *C. zeylanoides*	60	46.7^{a}	53.3^{a}	34.87

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

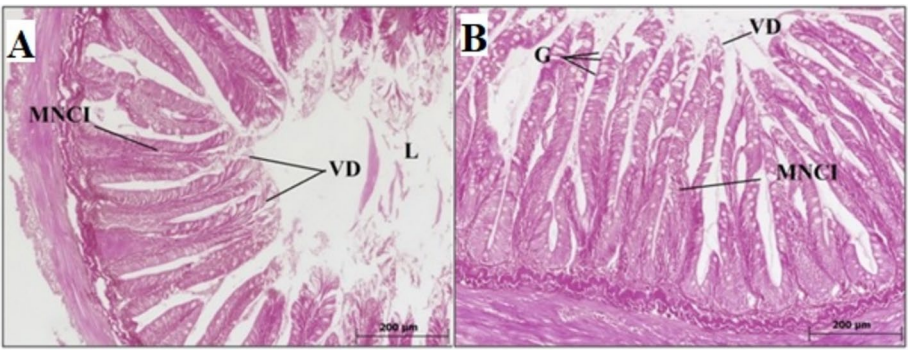

Fig. 1 Histology of proximal intestine, *C. zeylanoides* group (**A**) and *L. sakei* + *C. zeylanoides* group (**B**) (G, goblet cell; MNCI, mononuclear cell ınfiltration; VD, villus deformation; L, lumen), H&E

Challenge and survival rate

Regarding the challenge experiment, the survival rates in the *L. sakei* and *L. sakei* + *C. zeylanoides* groups were statistically significantly higher than the control group ($p < 0.05$). Although the survival rate in the *C. zeylanoides* group was higher than that in the control group, this difference was not statistically significant ($p > 0.05$). The highest RPS was found in the *L. sakei* group (Table 8).

Intestinal histology

No histological abnormalities were observed in the proximal intestinal sections of the control and *L. sakei* groups. In the *C. zeylanoides* group, partial deformations were noted in the apical parts of the villi (Fig. 1A). Moderate supranuclear lipid vacuolization and mononuclear cell infiltration were observed in the enterocytes, along with an increase in the number of goblet cells. In the *L. sakei* + *C. zeylanoides* group, partial deformations in the apical parts of the villi and an increase in goblet cell numbers were also observed in the proximal intestinal sections (Fig. 1B). Additionally, moderate supranuclear lipid vacuolization in enterocytes and mononuclear cell infiltration in the lamina propria of the villi were detected.

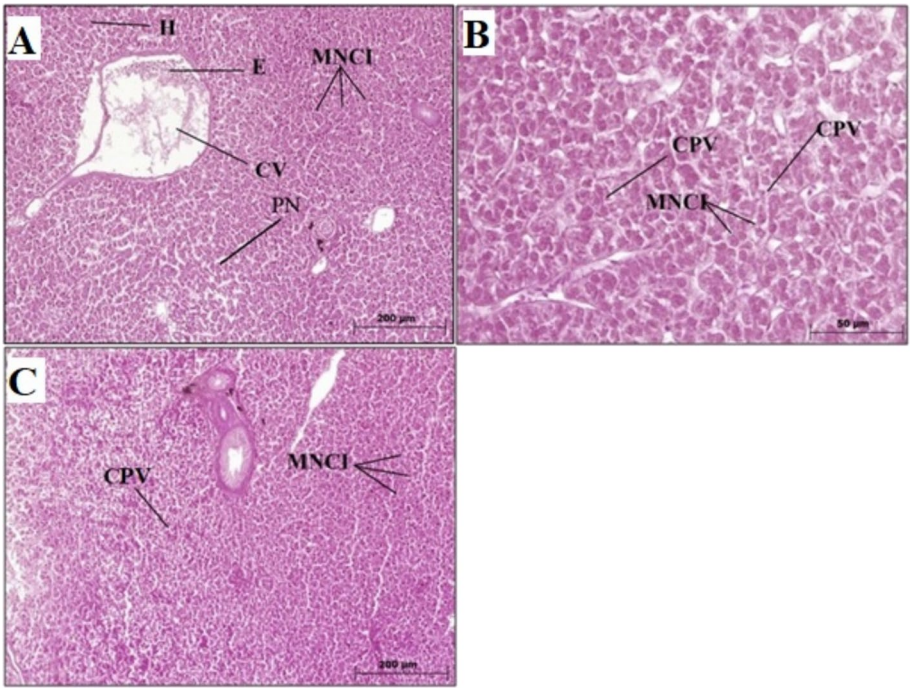

Fig. 2 Histology of liver, *L. sakei* group (**A**), *C. zeylanoides* group (**B**), and *L. sakei*+*zeylanoides* group (**C**) (MNCI, mononuclear cell ınfiltration; H, hepatocyte; E, erythrocyte; CV, central vena; CPV, cytoplasmic vacuolization; PN, pyknotic nuclei), H&E

Liver histology

No histological changes were observed in the liver sections of the control group fish. In the liver sections of fish fed with *L. sakei*, diffuse infiltrations and focal pyknotic nuclei in hepatocytes were noted (Fig. 2A). Focal cytoplasmic vacuolization was observed in the liver sections of fish fed with *C. zeylanoides* (Fig. 2B). In the liver sections of fish in the *L. sakei*+*C. zeylanoides* group, diffuse cytoplasmic vacuolization, cell infiltrations, and deformations in sinusoidal cavities were observed (Fig. 2C).

Histology of anterior kidney

Normal levels of melanomacrophage aggregations were observed in the anterior kidney sections in the control group (Fig. 3A). In the probiotic groups, both the quantity and size of melanomacrophage aggregations increased compared to the control group (Fig. 3B, C, and D).

The histological changes and their frequency of occurrence in the groups are presented in Table 9.

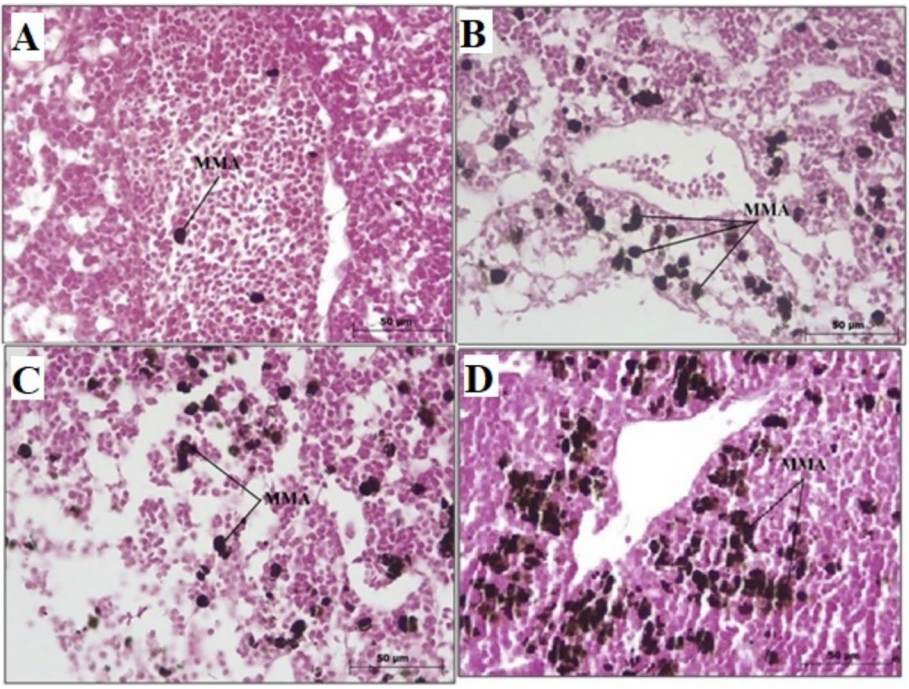

Fig. 3 Histology of anterior kidney, control group (**A**), *L. sakei* group (**B**), *C. zeylanoides* group (**C**), and *L. sakei* + *C. zeylanoides* group (**D**) (MMA, melanomacrophage aggregation), H&E

Table 9 Histological changes and their frequency were observed in control and probiotic groups (+, rare; + +, moderate; + + +, frequent)

Tissue	Histological change	Control	*L. sakei*	*C. zeylanoides*	*L. sakei* + *C. zeylanoides*
Proximal intestine	Supranuclear vacuolization	-	-	+ +	+ +
	Villus deformation	-	-	+	+ +
	Mononuclear cell infiltration	-	-	+ +	+ +
Liver	Hepatocyte vacuolization	-	+	+ +	+ +
	Mononuclear cell infiltration	-	+	+ +	+ +
	Pyknotic nuclei	-	+	+ +	+ +
Anterior Kidney	Melanomacrophage aggregation	+	+ +	+ + +	+ + +

Histomorphology of the proximal intestine

As a result of the experiment, the average number of goblet cells per villus was higher in the *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups compared to the control and *L. sakei* groups ($p < 0.05$) (Table 10).

Probiotics did not affect the villus length of the fish ($p > 0.05$). However, villus width was higher in the *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups compared to the control and *L. sakei* groups ($p < 0.05$) (Table 11).

Table 10 Goblet cells per villus of proximal intestine in control and probiotic groups

	Control	*L. sakei*	*C. zeylanoides*	*L. sakei* + *C. zeylanoides*
Goblet cells	9.8 ± 0.4^{b}	9.1 ± 0.6^{b}	14.5 ± 1.3^{a}	15.6 ± 1.7^{a}

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 11 Average villus length (VL) (μm), villus width (VW) (μm) in control and probiotic groups

	Control	*L. sakei*	*C. zeylanoides*	*L. sakei* + *C. zeylanoides*
VL (μm)	563.69 ± 140.16	578.88 ± 137.85	526.46 ± 117.8	521.65 ± 135.07
VW(μm)	43.31 ± 12.74[b]	41.22 ± 18.88[b]	65.63 ± 21.35[a]	56.53 ± 11.33[a]

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

Table 12 Hepatocyte diameters (HD) (μm) in liver sections of control and probiotic treatment groups (mean ± standard deviation)

	Control	*L. sakei*	*C. zeylanoides*	*L. sakei* + *C. zeylanoides*
HD (μm)	7.97 ± 1.06^{b}	8.15 ± 2.68^{b}	9.79 ± 2.6^{a}	9.9 ± 1.18^{a}

The values are presented as mean ± standard error. Different letters in the same column indicate statistical differences ($p < 0.05$).

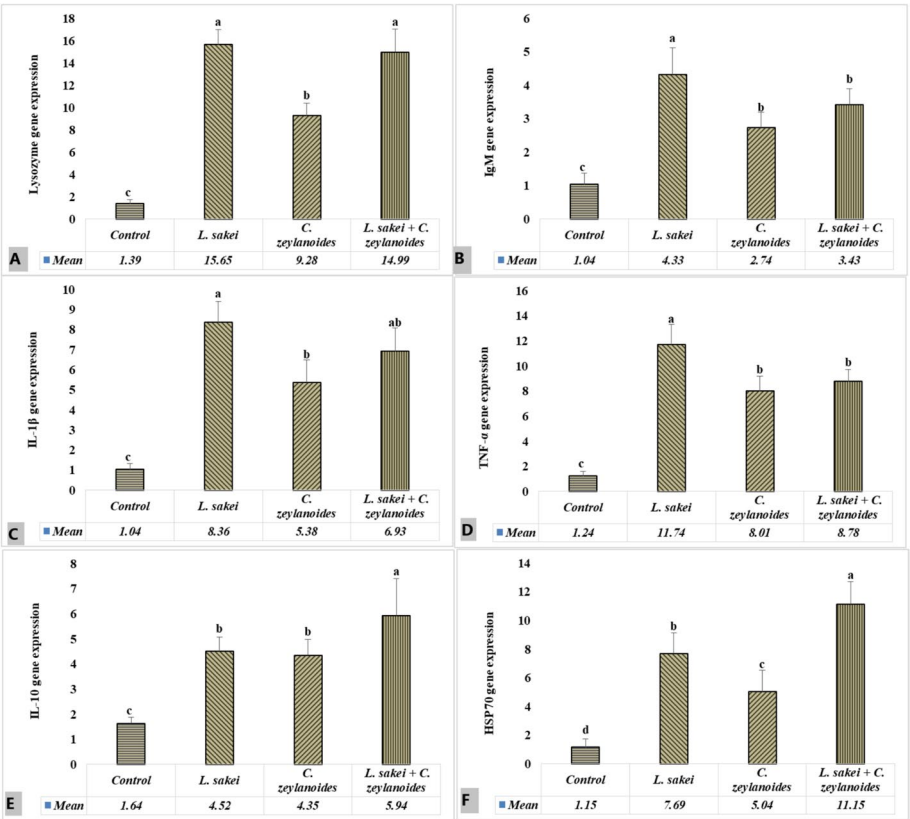

Fig. 4 Changes in the immune-related gene expression in the kidney: lysozyme (**A**), IgM (**B**), IL-1β (**C**), TNF-α (**D**), IL-10 (**E**), and HSP70 (**F**)

Histomorphology of liver

Hepatocyte diameters in the liver were enlarged in the *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups compared to the control and *L. sakei* groups ($p < 0.05$) (Table 12).

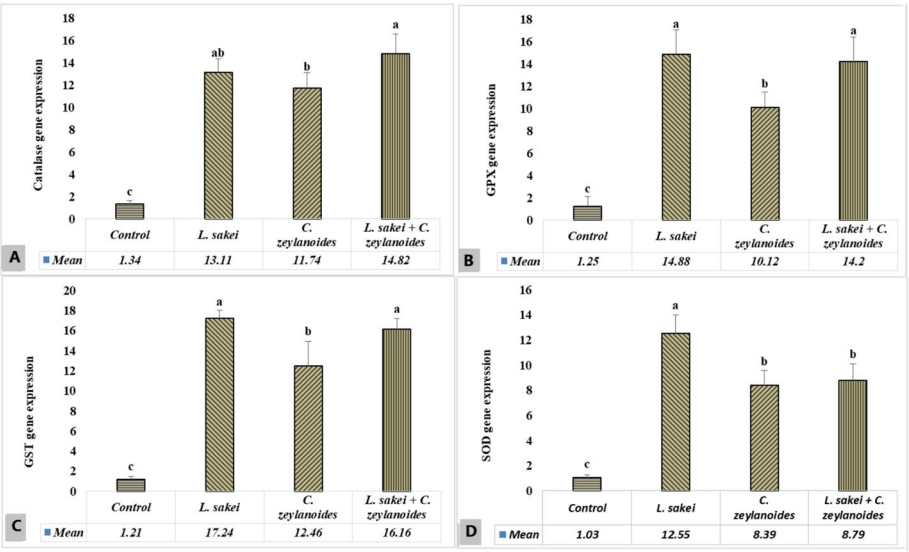

Fig. 5 Antioxidant enzyme genes expression in the liver: catalase (**A**), GPX (**B**), GST (**C**), and SOD (**D**)

Immune-related genes expression

As a result of the feeding experiment, expression levels of immune-related genes, including lysozyme, IgM, IL-1β, TNF-α, IL-10, and HSP70 in the anterior kidney enhanced in probiotic groups compared to the control group (Fig. 4A–F) ($p < 0.05$).

Antioxidant enzyme gene expression

After the feeding experiment, the expression of antioxidant enzyme genes in the liver of probiotic groups was higher compared to the control group (Fig. 5A–D) ($p < 0.05$). Additionally, the SOD gene expression in the liver of the *L. sakei* group was higher compared to the other probiotic groups ($p < 0.05$) (Fig. 5D).

Discussion

The study revealed a significant improvement in growth performance among fish-fed diets supplemented with probiotics. Ali et al. (2020) observed that adding *Lactobacillus farragi-nis* and *Enterococcus durans* strains, isolated from kefir, individually (10^8 cfu/g) into the diet of rainbow trout over 45 days improved both the specific growth rate and the feed conversion rate. Similarly, Alishahi et al. (2018) reported increased specific growth rate, weight gain, and daily weight gain in carp fed with *Lactobacillus bulgaricus*. Kong et al. (2020a) demonstrated that supplementing the diet of snakehead fish with *Lactococcus lac-tis* (L19) (1×10^8 cfu/g) and *Enterococcus faecalis* (W24) (1×10^8 cfu/g), both individu-ally and in combination (1×10^8 cfu/g + 1×10^8 cfu/g), for 56 days enhanced growth per-formance. Growth-promoting effects were also observed with *Lactobacillus delbrueckii*

(1×10^6 and 1×10^7 cfu/g of feed) in carp (Zhang et al. 2017), *Lactobacillus plantarum* (10^8 cfu/g of feed) in Pacific red snapper (Van Doan et al. 2014) and *Lactococcus lactis* (10^6 cfu/g of feed) and *Lactobacillus rhamnosus* (10^6 cfu/g of feed) in sea bream (Dawood et al. 2016). Shadrack et al. (2021) demonstrated that supplementing red sea bream diets with the yeast *Lipomyces starkeyi*, isolated from wheat straw, positively influenced specific growth rate and feed conversion efficiency. Additionally, Jahan et al. (2021) found that adding *Saccharomyces cerevisiae* to the diet of rohu fish improved growth performance and feed utilization. Likewise, Siddik et al. (2022) showed that supplementing barramundi (*Lates calcarifer*) diets with *S. cerevisiae* and *Lactobacillus casei* for 56 days enhanced final weight and growth performance.

After the feeding trial, fish that received *L. sakei* and *C. zeylanoides*, individually and in combination, exhibited increased serum lysozyme activity compared to the control group. A similar result was reported by Kong et al. (2020a), who observed that *L. lactis* L19 and *E. faecalis* W24, isolated from snakehead fish intestines, enhanced serum lysozyme levels. Ali et al. (2022) also noted that supplementing rainbow trout diets with *L. farraginis* and *E. durans*, both individually (10^8 cfu/g of feed) and in combination ($5\times10^6+5\times10^6$ cfu/g), increased serum lysozyme activity. Mohammadian et al. (2019) reported increased serum lysozyme activity in rainbow trout-fed *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* (5×10^7 cfu/g) for 60 days. Zhang et al. (2020a) observed a similar increase in lysozyme activity in carp intestines following an 8-week feeding trial with *L. delbrueckii* (1×10^6 and 1×10^7 cfu/g of feed). Several studies confirm that yeast strains can elevate serum lysozyme activity in fish. Çaylı Bektaş et al. (2024) found that adding *C. zeylanoides* Y12-3 and *H. pseudoburtonii* Y12-1 yeast strains (10^5 cfu/g of feed) to rainbow trout diets for 35 days increased serum lysozyme activity. Liao et al. (2022) demonstrated that the yeast *Metschnikowia sp.* GXUS03 enhanced lysozyme activity in tilapia. Hernández-Contreras et al. (2021) found that incorporating *Debaryomyces hansenii* CBS 8339 into yellowtail fish (*Seriola rivoliana*) diets increased lysozyme activity. Reyes-Becerril et al. (2017) observed that yeast *Sterigmatomyces halophilus* 04N-16 (1.1%) enhanced mucus lysozyme activity in sea bream. Similarly, Siddik et al. (2022) reported that *S. cerevisiae* and *L. casei* (1% yeast + 1% lactic acid bacteria) increased serum lysozyme activity in barramundi. However, Li and Gatlin (2003) found no change in serum lysozyme activity with brewer's yeast (*S. cerevisiae*) as a feed additive for hybrid striped bass. Boonanuntanasarn et al. (2019) also reported no effect on serum lysozyme levels when striped catfish were fed *S. cerevisiae* microcapsules.

In this study, serum myeloperoxidase activity did not change in the probiotics groups. Similarly, Çaylı Bektaş et al. (2024) found no effect on serum myeloperoxidase levels after feeding with *C. zeylanoides* Y12-3 and *H. pseudoburtonii* Y12-1 yeast strains (10^5 cfu/g of feed for 35 days) in rainbow trout. Reyes-Becerril et al. (2011) also observed no significant changes in serum myeloperoxidase activity in leopard grouper (*Mycteroperca rosacea*) after a 4-week feeding trial with the yeast strain *D. hansenii*CBS 8339. Likewise, Hernández-Contreras et al. (2021) found that *D. Hansenii* CBS 8339, isolated from the intestines of rainbow trout, did not affect myeloperoxidase activity in mucus in yellowtail fish (*S. rivoliana*). Phinyo et al. (2024) reported no changes in the serum myeloperoxidase levels in Nile tilapia fed *Lactococcus lactis* TISTR 1464 (10^8 cfu/g of feed for 10 weeks). However, some studies suggest that yeast or lactic acid bacteria strains can increase myeloperoxidase activity. For example, Reyes-Becerril et al. (2017) found that the yeast *S. halophilu* 04N-16 (0.55% feed) increased myeloperoxidase activity in sea bream mucus. Alamillo et al. (2017) also reported that the yeast *Yarrowia lipolytica* N-6 enhanced myeloperoxidase activities in leukocytes from the anterior kidney of Pacific red snapper.

Zhang et al. (2020a) similarly found that myeloperoxidase enzyme levels in carp intestines increased following an 8-week feeding trial with *L. delbrueckii* (1×10^6 and 1×10^7 cfu/g of feed). Lee et al. (2013) observed that *Lactobacillus pentosus* PL11 (10^8 cfu/g of feed), used for 5 weeks, elevated serum myeloperoxidase levels in eel (*Anguilla japonica*). Won et al. (2020) reported that *Lactococcus lactis* supplementation (1×10^7 and 1×10^8 cfu/g of feed) for 8 weeks increased myeloperoxidase activity in Nile tilapia. The different results between these studies could be attributed to differences in fish species, probiotic strains, or feeding durations.

In the present study, fish fed the *L. sakei* demonstrated higher respiratory burst activity than the control group. Maji et al. (2017) also observed increased respiratory burst activity in Indian carp (*Labeo rohita*) following the use of *Lactobacillus plantarum* SM16, *L. plantarum* SM33, *L. fermentum*, *L. brevis* SM56, and *Pediococcus pentosaceus* SM64, isolated from freshwater fish intestines, in their diet. Nikoskelainen et al. (2003) reported increased respiratory burst activity in rainbow trout fed with *Lactobacillus rhamnosus* (7.9×10^4 cfu/g of feed) for 2 weeks.

Increased serum glucose, total protein, albumin, and globulin levels in fish are indicators of improved overall health (Eissa et al. 2022). In our study, the serum glucose concentrations were significantly higher in probiotic groups compared to the control group. Several studies demonstrate that yeast or lactic acid bacteria strains can increase serum glucose levels in fish. Çaylı Bektaş et al. (2024) found that the yeast *H. pseudoburtonii* Y12-1 increased glucose levels in rainbow trout. Similarly, Abdel-Tawwab et al. (2008) reported that adding live baker's yeast *S. cerevisiae* at 0.025%, 0.5%, and 1% to tilapia diets elevated serum glucose levels. Mehdinejad et al. (2018) found increased total serum glucose levels in goldfish (*Carassius auratus*) fed with *Pediococcus acidilactici* (0.2%). However, some studies have reported that supplementing fish diets with yeast or lactic acid bacteria does not affect glucose levels. Hoseinifar et al.(2011) noted no change in glucose levels in sturgeon (*Huso huso*) fed 1% and 2% inactive brewer's yeast (*S. cerevisiae* var. *ellipsoideus*). Similarly, Boonanuntanasarn et al. (2019) found no effect on serum glucose levels in striped catfish fed with *S. cerevisiae* microcapsules. Eissa et al. (2022) also reported no changes in sea bass-fed commercial probiotics (Pediococcus acidilactici) serum glucose levels at 2, 2.5, and 3 g/kg doses. Differences in results may be due to differences in fish species and probiotic strains.

In our study, total protein and globulin levels were higher in fish fed with *L. sakei* 2–3 than in the control group. Similarly, Mehdinejad et al. (2018) reported increased total protein and albumin levels in goldfish fed 0.2% *Pediococcus acidilactici*. On the other hand, Eissa et al. (2022) noted no changes in total protein and globulin levels in sea bass fed with *Pediococcus acidilactici* at doses of 2, 2.5, and 3 g/kg. These differences may be due to differences in fish species and probiotic strains.

In the present study, serum cholesterol levels were unaffected by probiotic supplementations. Likewise, Boonanuntanasarn et al. (2019) found that feeding striped catfish with microencapsulated *S. cerevisiae* did not influence serum cholesterol levels. Hoseinifar et al. (2011) also observed no effect on serum cholesterol levels in sturgeon (*Huso huso*) fed with 1% and 2% inactive brewer's yeast (*S. cerevisiae* var. *ellipsoideus*). There is limited research on lactic acid bacteria's impact on fish's cholesterol levels. Piccolo et al. (2015) reported that L. plantarum caused an increase in total cholesterol value in European sea bass. However, Kong et al. (2020b) demonstrated that the *Lactococcus lactis* L19 strain showed strong cholesterol assimilation capabilities in vitro.

In this study, *C. zeylanoides* Y12-3 reduced serum triglyceride levels. Çaylı Bektaş et al. (2024) reported that *C. zeylanoides* Y12-3 reduced triglyceride levels in rainbow trout at a

feed dose of 10^5 cfu/g for 35 days. Hassaan et al. (2018) also observed that *S. cerevisiae* (10 and 15 g/kg of feed) decreased serum triglyceride levels in Nile tilapia. In contrast, Çaylı Bektaş et al. (2024) reported no effect of *H. pseudoburtonii* Y12-1 on triglyceride levels in rainbow trout. Similarly, Boonanuntanasarn et al. (2019) found no effect on serum triglyceride levels in striped catfish fed microencapsulated *S. cerevisiae*.

Liver enzymes, specifically ALT and AST, are key liver function and health indicators. Monitoring changes in these enzymes is essential for assessing the effects of feed additives on metabolic activity and overall fish health, as increased levels of ALT and AST may signal liver cell damage (Eissa et al. 2022). In our study, the AST level in the *L. sakei 2–3* + *C. zeylanoides 12–3* group was lower than the control group. Nevertheless, there was no change in ALT levels in the probiotic groups. Similarly, Yeganeh Rastekenari et al. (2021) found no changes in ALT levels in sturgeon fry fed *L. lactis* and *Weissella confusa*.

Histopathological examination in this study revealed that fish fed with *C. zeylanoides* and *L. sakei* + *C. zeylanoides* exhibited supranuclear vacuolization, villus deformation, and mononuclear cell infiltration in the intestines; hepatocyte vacuolization, mononuclear cell infiltration, and pyknotic nuclei in the liver; and melanomacrophage aggregation in the anterior kidney. Similarly, Aly et al. (2024) observed mild vacuolization in liver cells, melanomacrophage aggregation around hepatopancreatic regions, minimal kidney degeneration, increased melanomacrophage centers within the renal parenchyma, proliferation, and activation of these centers throughout the splenic parenchyma, and mucinous degeneration in the intestinal epithelium of Nile tilapia treated with *S. cerevisiae*.

Melanomacrophage centers can indicate an enhanced immune response in the liver (Agius 1979; Agius and Roberts 2003). This suggests that probiotics with immunomodulatory properties can support liver health through their anti-inflammatory effects (Yousefiet al. 2019; Zhang et al. 2023). In this study, histological analysis of kidney tissues showed an increase in the amount and volume of melanomacrophage aggregation in the anterior kidney in the probiotic groups compared to the control group. Similarly, Shehata et al. (2024) found that the application of *Lactobacillus bulgaricus* and *L. bulgaricus* + *S. cerevisiae* increased melanomacrophage centers near the hepatic central veins in the liver of *Mugil capito*.

Regarding the histomorphology of the proximal intestines, villus length was not influenced by probiotic supplementation in this study. However, villus width and the number of goblet cells in fish fed with *C. zeylanoides*Y12-3 and *L. sakei* 2–3 increased compared to the control and *L. sakei* groups. Similarly, Siddik et al. (2022) reported that intestinal villus width and goblet cell numbers increased in barramundi fed *S. cerevisiae* + *L. casei* (1% yeast + 1% lactic acid bacteria) for 56 days. Jahan et al. (2021) also observed increased villus width in rohu fish fed with *S. cerevisiae*.

In our study, the expression of immunity-related genes (IgM, IL-B1, IL-10, lysozyme, TNF-α, HSP70) in the kidney and genes related to antioxidant enzyme (catalase, GPX, GST, SOD) in the liver were increased in probiotic groups. Similarly, Kong et al. (2020a) used *Lactococcus lactis*(L19) and *E. faecalis* (W24) isolated from the intestines of snakehead fish and reported significant upregulation of TNF-α, IL-6, IFN-γ, IL-10, IL-1β, TGF-β, HSP90, and HSP70 genes in the spleen, anterior kidney, gill, liver, and intestines. Pérez-Sánchez et al. (2011) also found that supplementing rainbow trout diets with *L. plantarum* (10^6 cfu/g) for 36 days enhanced the expression of immune-related genes (IL-10, IL-8, and IgT). Guo et al. (2020) similarly observed that adding *Leuconostoc mesenteroides* (10^5 cfu/ml) to the rearing water of turbot increased the expression of immune-related genes (TLR3, IL-8, and IFIH1) in internal organs. Ali et al. (2020) noted that *L. farraginis* and *E. durans* strains (10^8 cfu/g) significantly increased the expression of lysozyme and TNF-α genes in

the intestinal tissues of rainbow trout. Maji et al. (2017) reported that the expression of the IL-10 gene in the kidney, intestine, and liver, and the TNF-α gene in the liver and intestine of Indian carp was significantly upregulated by the use of *Lactobacillus plantarum* SM16, *L. plantarum* SM33, *L. fermentum* SM51, *L. brevis* SM56, and *Pediococcus pentosaceus* SM64. Siddik et al. (2022) similarly observed that supplementing the diet of barramundi with a combination of *S. cerevisiae* and *L. casei* (1% yeast + 1% lactic acid bacteria) for 56 days resulted in increased expression of the IL-10 and TNF-α genes. Vazirzadeh et al. (2020) demonstrated that the expression of the TNF-α gene in the spleen of rainbow trout increased after long-term feeding (130 days) with the *S. cerevisiae* strain (10^7 cfu/feed). Beck et al. (2015) found that *L. lactis* BFE920 and *L. plantarum* FGL0001 (10^7 cfu/g of feed) enhanced the expression of IL-6, IL-8, and TNF-α genes in the intestines of flounder (*Paralichthys olivaceus*).

Reyes-Becerril et al. (2008) demonstrated that feeding sea bream with *Debaryomyces hansenii* CBS 8339 (10^6 cfu/g of feed) for 4 weeks resulted in an enhanced expression of immune-related genes (IgM, MHC-IIα, TCR-β, TNF-α, CSF-1R, IL-1β, and NCCRP-1) in the liver, intestine, and anterior kidneys. Similarly, Reyes-Becerril et al. (2011) reported that using *D. hansenii* CBS 8339 (10^6 cfu/g of feed) for 5 weeks in juvenile leopard grouper enhanced the expression of the HSP70 gene. In another study, Reyes-Becerril et al. (2012) observed that *D. hansenii* L2 (10^6 cfu/g of feed) upregulated the expression of IgM, C3, TLR, MHCIIα, MHCIα, TNFα, CSF-1R, and IL-1β genes in the skin, intestine, and anterior kidney in sea bream. Reyes-Becerril et al. (2021) also noted that adding *D. hansenii* BCS004 to the diet of sea bream for 4 weeks increased the expression of TNF-α, C3, and IgM genes in intestinal samples.

In this study, *L. sakei* and the combination of *L. sakei* + *C. zeylanoides* provided resistance to *L. garvieae* compared to the control group. However, *C. zeylanoides* was similar to the other two probiotic groups regarding disease resistance against *L. garvieae*. Previous studies also reported *Leuconostoc mesenteroides* CLFP 196 and *L. plantarum* (Vendrell et al. 2006), *Lactococcus cremoris* (Araújo et al. 2015), and *Lactococcus lactis* subsp. *lactis* M17 2–2, *Lactobacillus sakei* 2–3 (Didinen et al. 2018), *Enterococcus faecalis* UGRA10 (Baños et al. 2019), *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus, L. farraginis* (Mohammadian et al. 2019), and *E. durans* (Ali et al. 2022) provided disease resistance against lactococcosis caused by *L. garvieae* in rainbow trout. Additionally, Çaylı Bektaş et al. (2024) noted that feeding rainbow trout with yeast strains *H. pseudoburtonii* Y12-1 and *C. zeylanoides* Y12-3 (10^5 cfu/g of feed) provided disease resistance against *L. garvieae*. Similarly, Li and Gatlin (2003) also showed that hybrid striped bass fed *S. cerevisiae* exhibited disease resistance to *Streptococcus iniae* infection. Liao et al. (2022) also reported that the yeast *Metschnikowia sp.* GXUS03 provided disease resistance against *Streptococcus agalactiae* in Nile tilapia.

Conclusions

According to the result of this study, supplementation with *L. sakei* 2–3 and *C. zeylanoides* Y12-3 strains (individually or in combination) increased growth parameters, serum glucose levels, serum lysozyme activity, and expression of immune and antioxidant enzyme genes compared to the control group. In addition, triglyceride levels were reduced in the probiotic groups, while serum ALT levels remained unchanged. The *L. sakei* 2–3 strain showed higher superoxide anion production, expression of immunity-related genes (IgM, IL-B1, lysozyme, TNF-α, HSP70), and antioxidant enzyme genes (GPX, GST, SOD) compared to *C.*

zeylanoides. *L. sakei* and the combination of *L. sakei* + *C. zeylanoides* provided resistance to *L. garvieae* compared to the control group. However, *C. zeylanoides* was similar to the other two probiotic groups regarding disease resistance against *L. garvieae*. Histopathological examinations revealed some reversible changes in the proximal intestine, anterior kidney, and liver in the *C. zeylanoides* and *L. sakei* + *C. zeylanoides* groups. Hence, future studies are still required to explore the effects of shorter-term use of the *C. zeylanoides* strain in rainbow trout to prevent undesirable effects on tissues. In brief, the abovementioned findings showed that *L. sakei* 2–3 and *C. zeylanoides* could be potential probiotic candidates for use in rainbow trout farming. Moreover, both probiotic strains in different fish species should also be studied.

Acknowledgements This study is supported by TUBITAK (The Scientific and Technological Research Council of Turkey) Project with Number 123O011. We would like to thank TUBITAK for its support.

Author contribution Mohanad Dheyaa Taha: methodology, investigation, validation, data curation, writing–originaldraft, review and editing. Behire Işıl DİDİNEN: methodology, investigation, writing–originaldraft, review and editing. Sevdan YILMAZ: methodology, review and editing. Mert GÜRKAN: methodology. Osman Nezih KENENOĞLUe: methodology.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). This study is supported by the TUBITAK (The Scientific and Technological Research Council of Turkey) Project with Number 123O011.

Data availability Data will be available upon request.

Declarations

Ethics approval This study was approved by the local ethics committee for animal experiments of Çanakkale Onsekiz Mart University, Çanakkale, Turkey (Approval No:2022/11–10).

Conflict of interest The authors declare no competing interests.

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