Investigating the Effect of *Lactobacillus plantarum* and *Lactobacillus fermentum* Probiotics on the Expression of *tdh* and *trh* Genes of *Vibrio parahaemolyticus* Isolated from Farmed Fish using real-time PCR

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Abstract

Introduction

Effective disease management in aquaculture depends largely on preventive strategies and enhancement of the fish immune system. Farmed fish represent a major portion of seafood consumption in Iran. However, limited information is available regarding the prevalence of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) strains harboring enterotoxin genes and the potential mitigating effects of probiotics in Iranian aquaculture systems. Considering the pathogenic potential of this bacterium and its impact on public health, updated data are essential.

Objective

The present study aimed to investigate the occurrence of *V. parahaemolyticus* in farmed carp and trout in Tehran, evaluate the presence of enterotoxin-related genes, determine antibiotic susceptibility patterns, and assess the inhibitory and gene expression–modulating effects of two probiotic species, *Lactobacillus fermentum* (*L. fermentum*) and *Lactobacillus plantarum* (*L. plantarum*).

Material and Methods

A total of 264 fish samples, including carp and trout, were collected from fish farms in Tehran. Isolation of *V. parahaemolyticus* was performed using enrichment media followed by standard biochemical identification tests. Molecular confirmation of isolates and detection of enterotoxin-associated genes were carried out using Polymerase Chain Reaction (PCR). Antibiotic susceptibility testing was conducted to determine resistance patterns. The antagonistic activity of *L. fermentum* and *L. plantarum* against the isolates was evaluated using in vitro assays. Furthermore, the impact of these probiotics on enterotoxin gene expression was analyzed through real-time PCR.

Results

Out of 264 examined fish samples, 20 (7.57%) were positive for *V. parahaemolyticus*, including 14 carp (10.6%) and 6 trout (4.54%). All isolates carried the 16S rRNA gene, confirming species-level identification. The *tdh* gene, a key virulence marker, was detected in 16 isolates (80%), while the *trh* gene was not identified in any samples. Both probiotic strains exhibited significant inhibitory effects on bacterial growth in vitro. Real-time PCR analysis demonstrated a considerable reduction in *tdh* gene expression following exposure to either *L. fermentum* or *L. plantarum*.

Conclusion

The detection of the *tdh* virulence gene in *V. parahaemolyticus* isolates from farmed fish highlights the necessity for continuous surveillance and strengthened biosecurity measures in aquaculture systems. The demonstrated inhibitory and gene expression–reducing effects of the tested probiotics suggest their potential as alternative preventive strategies in aquaculture. Additionally, strict hygiene practices during fish handling, thorough washing, and proper cooking are recommended to reduce potential public health risks.

Keywords: Aquaculture species, Foodborne pathogens, *Vibrio parahaemolyticu*, Virulence genes

1. Introduction

Fish and seafood represent the second most consumed sources of animal protein worldwide, following red meat and poultry; notably, in nations such as Japan, fish constitutes the primary source of dietary protein [1]. *V. parahaemolyticus* is a naturally occurring bacterium prevalent in the microbial flora of coastal waters and rivers. Among the pathogenic bacteria associated with seafood, *V. parahaemolyticus* is the most frequently encountered [2]. It belongs to the Vibrionaceae family, is non-spore-forming, exhibits motility due to its polar flagellum, and is a gram-negative bacillus that displays halophilic characteristics, necessitating 1 to 3% salt for optimal growth, yet capable of enduring salinities up to 7% [2,3]. The global distribution and prevalence of *V. parahaemolyticus* are strongly influenced by environmental factors such as seasonal temperature variations, water salinity, and fecal contamination [1]. The infectious dose is generally estimated to exceed 10^6 cells per gram of food, with an incubation period ranging from 3 to 76 hours and clinical symptoms lasting between 1 and 8 days [1,3]. This pathogen is commonly isolated from a variety of seafood products, including fish, shrimp, crab, lobster, and oysters. Consumption of raw or undercooked contaminated seafood may lead to acute gastroenteritis, characterized by diarrhea, nausea, vomiting, abdominal cramps, headache, and mild fever [1].

Strains of *V. parahaemolyticus* isolated from patients experiencing gastroenteritis and acute diarrhea typically produce either the thermostable direct hemolysin (TDH), the TDH-dependent hemolysin (TRH), or both [1]. The TDH factor operates as an amyloid toxin that disrupts the lipid bilayers of host cells and mitigates immune system-induced toxicity in bacterial cells. Additionally, it may enhance the uptake of iron ions into bacterial cells. Although the correlation between the presence of TDH and TRH factors and the pathogenicity of *V. parahaemolyticus* is well documented, numerous clinical strains are noted to lack either the TDH or TRH factors [4].

Seafood is consumed globally as a nutritious and healthful food source. As the consumption of seafood rises, the health implications associated with it are undergoing increased scrutiny [1]. To

control diseases in aquaculture, an effective strategy involves enhancing aquatic defense mechanisms through preventive measures and the stimulation of the immune system [2,3]. Common immune stimulants in aquaculture include probiotics, prebiotics, and their combination, referred to as synbiotics. These microorganisms are incorporated into the diet to modify the intestinal bacterial flora, thereby reducing harmful microbial populations within the digestive system [5]. By implementing microbial management with beneficial bacteria, compatibility between the host and its breeding environment is improved, leading to enhanced utilization of water resources and increased production efficiency. These compounds function not only as growth stimulants but also as immune enhancers that aid in disease prevention by promoting a balanced intestinal microbiome [3,5].

Although the adoption of probiotics within the aquaculture industry remains limited, their advantages include resilience to elevated temperatures during pellet production and an extended shelf life. The combined benefits of probiotics and prebiotics include competition with pathogenic bacteria for resources, stimulation of appetite, and the degradation of indigestible dietary components via protease and amylase enzymes [1, 5]. This results in enhanced growth, survival, and immunity in the host through the production of vitamins such as riboflavin and K. Consequently, improvements in immune function, increased disease resistance, reduced stress, higher survival rates, enhanced growth indices, improved feed efficiency, and superior meat quality are observed [6]. The escalating costs associated with wild fish have instigated a transformation in aquatic consumption patterns, leading to a predominance of consumers opting for more economically accessible and readily available farmed species, notably trout and carp [1].

While numerous investigations in Iran and other countries have focused on *V. parahaemolyticus* isolated from marine organisms, research exploring the effects of probiotics on *V. parahaemolyticus* strains harboring enterotoxin genes remains limited [5]. Acknowledging the importance of this issue, we undertook a study examining two probiotics, *L. plantarum* and *L. fermentum*, which are recognized for their capacity to inhibit a range of intestinal pathogens [5, 3]. Our primary objective was to assess their influence on the growth of *V. parahaemolyticus* and the expression of the *tdh* and *trh* enterotoxin genes in farmed fish obtained from Tehran.

2. Materials and Methods

A total of 264 fish samples, specifically comprising trout and carp, were randomly acquired under sanitary conditions from aquatic distribution areas in Tehran. Fresh samples were transported to the Zoonosis Research Center at Tehran University of Medical Sciences within 24 hours of collection, while maintaining a temperature of 4°C. The samples were cultured and examined under sterile conditions, adhering to standard protocols for isolating Vibrio from food.

Under aseptic conditions, 25 grams of abdominal muscle were extracted from each sample and incubated in 225 milliliter of alkaline peptone water for 24 hours at 37°C. A loopful of the culture was subsequently inoculated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) medium (Germany, Merck). Following overnight re-incubation, suspected colonies were evaluated to confirm the presence of *V. parahaemolyticus*. Various tests, including the oxidase test (England, Mast), Gram stain (Bahar Afshan, Iran), carbohydrate fermentation, citrate, motility, urease, arginine hydrolysis, and Voges-Proskauer (VP) tests (Germany, Merck), were conducted for this confirmation. The *V. parahaemolyticus* strain ATCC187028 served as a positive control [3].

2.1 Molecular Identification of *V. parahaemolyticus* Isolates and Investigation of Enterotoxin Genes

2.1.1 DNA Extraction

The boiling method utilizing the BIOER thermo block was employed for DNA extraction from isolates phenotypically identified as *V. parahaemolyticus*. The quality and concentration of the extracted DNA were assessed using the THERMO Nano drop device. For molecular confirmation, the 16S rRNA gene specific to *V. parahaemolyticus* was targeted, alongside the analysis of virulence genes *tdh* and *trh*. Primers were obtained from reputable literature, re-blasted, and synthesized by Pishgam Company in Germany [3]. The characteristics of the studied primers are detailed in (Table 1).

Table1. Characteristics of the studied primers

The Studied Gene	Primer Synthesis (5′ → 3′)	Product size (base pair)	References
16 SrRNA	F:GCAGCTGATCAAAACGTTGAGT R:ATTATCGATCGTGCCACTCAC	842	**3**
tdh	F:GTAAAGGTCTCTGACTTTTGGAC R:TGGAATAGAACCTTCATCTTCACC	269	**9**
trh	F:TTCACAAAATCAGAAAAAACAAGA R:TTTAATTTTGTGACATACATTCATC	217	**9**

An Eppendorf PCR machine (USA) was utilized to perform PCR in a final volume of 20 microliters. Image detection was achieved using a gel doc device, with the final PCR product subjected to electrophoresis in 1% agarose in 0.5X TBE solution.

2.2 Assessment of Antibiotic Resistance in *V. parahaemolyticus*

The antibiotic resistance of confirmed *V. parahaemolyticus* was evaluated using the agar disk diffusion method, in accordance with the guidelines established by CLSI 2025. The antibiotics (Mast.UK) tested included [7]:

- Chloramphenicol (30 µg)
- Levofloxacin (5 µg)
- Ciprofloxacin (5 µg)
- Tetracycline (30 µg)
- Clavulanic acid (30 µg)
- Gentamicin (30 µg)
- Imipenem (10 µg)
- Doxycycline (30 µg)
- Ceftazidime (30 µg)
- Co-trimoxazole (30 µg)

2.3 Investigation of the Antimicrobial Activity of *L. fermentum* and *L. plantarum* on *V. parahaemolyticus*

To examine the antimicrobial activity of *L. fermentum* and *L. plantarum*, the well diffusion method was employed. Two strains, *L. plantarum* PTCC 8014 and *L. fermentum* PTCC 9338 were procured from the Microbial Strain Collection Center (MSCC).

The supernatant from a 24 h culture of these lactobacilli in De Man–Rogosa–Sharpe (MRS) (Germany, Merck) liquid medium was separated by centrifugation at 13000 Revolutions Per Minute (RPM) for ten minutes. A turbidity equivalent to the 0.5 McFarland standard was prepared from a fresh culture of *V. parahaemolyticus* in Luria-Bertoni (LB) liquid medium (Merck, Germany). The bacteria were cultured as a lawn on Mueller Hinton agar (Merck, Germany) from the prepared standard, and wells were created. One hundred microliters of the filtered (0.45 μm) supernatant from the lactobacillus culture were added to each well of a Mueller Hinton agar plate, which was then incubated at 37°C for 18 to 24 hours. After incubation, the diameter of the zone of inhibition surrounding the wells was measured. Each test was repeated three times for each *V. parahaemolyticus* isolate to ensure the reliability of the results [3].

2.4 Determination of the MIC of Culture Supernatant from *L. fermentum* and *L. plantarum* Strains

The MIC of the culture supernatant derived from *L. fermentum* and *L. plantarum* strains was ascertained utilizing the microdilution broth method within a 96-well microplate format. Each well was inoculated with 100 μl of MRS broth culture medium. Subsequently, 100 μl of the culture supernatant from the lactobacillus strains was introduced into the first well, followed by a serial dilution, producing final concentrations of the culture supernatant in the wells ranging from 1 to $\frac{1}{512}$. Following this, 100 μl of a cell suspension from each *V. parahaemolyticus* isolate, diluted to 1.5×10^6 CFU/ml in Mueller Hinton broth was added to each well. This procedure was replicated three times, and the 96-well plate was incubated at 37°C for duration of 24 hours. Post-incubation, the growth of *V. parahaemolyticus* strains was evaluated by measuring the turbidity of the wells using a Biotek ELX800 ELISA plate reader [8].

RNA extraction from the bacterial suspension subjected to the MIC of probiotics was conducted utilizing an RNA extraction kit (Cat No: YT9080) provided by Yekta Tajhiz Azma Company. The synthesis of complementary DNA (cDNA) from the extracted RNAs was performed employing a cDNA synthesis kit (Cat No: YT4500) from the same company.

2.5 Real-time PCR and Examination of *tdh* and *trh* Gene Expression

The real-time PCR reaction was designed to investigate the expression levels of the *tdh*, *trh*, and 16S rRNA genes following the application of each examined probiotic on *V. parahaemolyticus* bacteria that possess these genes, in addition to control samples. The Step One Plus Real-time PCR device and SYBR Green qPCR Master Mix 2X laboratory kit (Cat No: YT2551) from Yekta Tajhiz Azma Company were employed. Primer sequences for these genes were developed using Gene Runner software. Additionally, another 16S rRNA gene with the sequence F: TATCCTTGTTTGCCAGCGAG and R: CTACGACGCACTTTTTGGGT, corresponding to a molecular weight of 186 bp, was utilized as a reference gene [9].

2.6 Statistical Analysis of the Expression Levels of the Studied Genes

The quantitative assessment of gene expression levels via real-time PCR was executed employing two methodologies: absolute quantification and relative quantification. Absolute quantification determined the precise number of copies of the studied gene, while relative quantification compared the expression level of the target gene to that of a reference gene. Prism 9 GraphPad software was utilized for statistical analysis of the data. The expression level of the *tdh* gene (samples examined were lacking the presence of the *trh* gene) in strains treated with the investigated probiotics was compared to that in control strains based on $2^{-\Delta Ct}$. The significance of the data was evaluated utilizing the T-test [10].

3. Results

In this study, 20 out of 264 farmed fish samples (7.57%) were identified as infected with *V. parahaemolyticus*. Among these, 14 samples (70%) originated from carp, while 6 samples (30%) were derived from trout. The prevalence of other Vibrio species isolated from farmed fish is summarized in (Table 2).

Table2. Number and percentage of other Vibrio species isolated by farmed fish

Vibrio Species	Carp Number (%)	Trout Number (%)
V. vulnificus	1 (0/75)	2 (1/5)
V. fluvialis	1 (0/75)	0 (0)
V. mimicus	6 (4/54)	5 (3/78)
V. alginolyticus	2 (1/5)	2 (1/5)
V. damsela	5 (3/78)	7 (5/30)

3.1 Results of Antibiotic Susceptibility and Resistance Testing for *V. parahaemolyticus* Isolates

Antibiotic susceptibility and resistance testing of *V. parahaemolyticus* isolates demonstrated the highest sensitivity to ciprofloxacin (83.33%) and chloramphenicol (77.78%). Resistance was most pronounced against amoxicillin and tetracycline, with both exhibiting 100% resistance. Other isolated Vibrio species also exhibited high sensitivity to ciprofloxacin (87.09%) and 100% resistance to amoxicillin, ceftazidime, and meropenem.

3.2 Frequency Results of *tdh* and *trh* Genes

All isolates identified as *V. parahaemolyticus* through phenotypic observation and biochemical tests were confirmed via the specific 16S rRNA gene associated with the flagellum of *V. parahaemolyticus*. Sixteen isolates (80%) contained the *tdh* gene, whereas the *trh* gene was not detected in any samples (Figure 1).

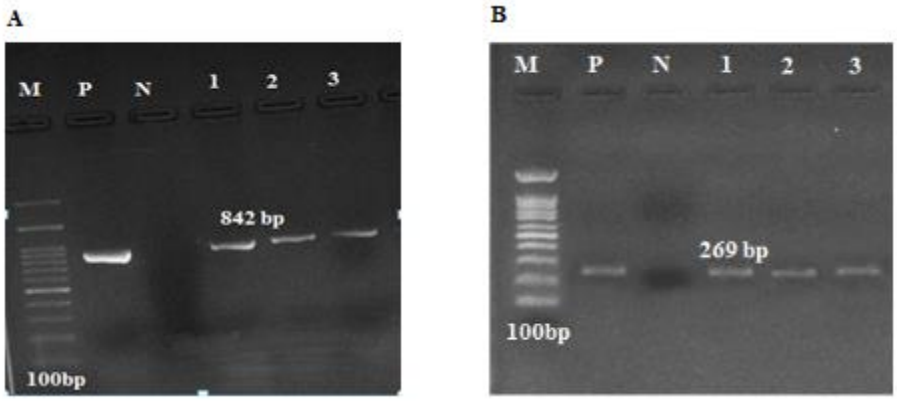


Figure 1. Electrophoresis results of the gene. A: Flagellar 16S rRNA, Product size: 842 bp. B: *tdh* gene, Product size: 269 bp.

M: 100 bp Marker, P: Positive control *V. parahaemolyticus* strain ATCC 33846, N: Negative control (distilled water), 16S rRNA and *tdh* gene primers, Mastermix, 1, 2, and 3: Tested samples containing the gene of interest.

3.3 Results of the Study on the Inhibitory Effect of Probiotic Supernatant on *V. parahaemolyticus*

The antimicrobial effect of probiotic lactobacillus liquid on inhibiting *V. parahaemolyticus* growth, assessed using agar well and agar spot methods, exhibited significant inhibitory activity. The MIC of the culture supernatants from *L. fermentum* and *L. plantarum* strains were $\frac{1}{64}$ and $\frac{1}{128}$, respectively. The results of the growth inhibition effect are depicted (Figure 2).

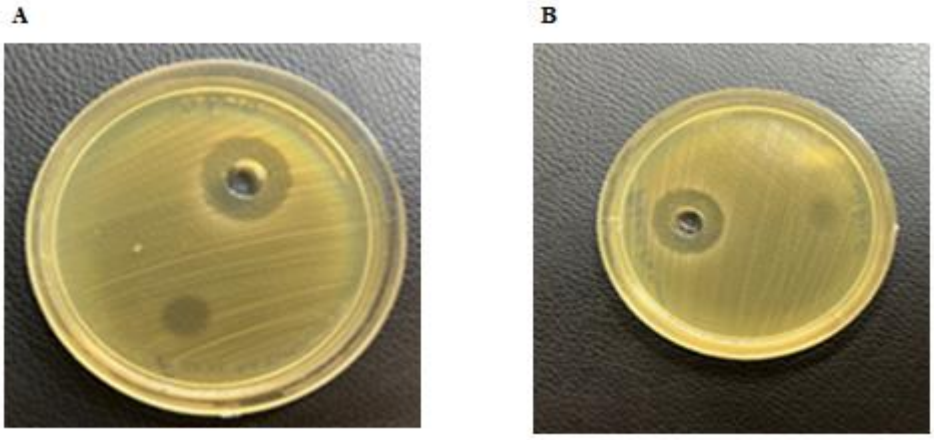


Figure 2. Inhibition of *V. parahaemolyticus* growth by A: *L. fermentum* PTCC9338 and B: *L. plantarum* PTCC 8014, assessed using the well-plate and agar spot methods following a 24 hour incubation period in a conventional incubator at 37 °C on Mueller Hinton agar medium.

3.4 Results of 16S rRNA Gene Expression Study Utilizing the Cyber Green Real-Time PCR Technique

In this investigation, the 16S rRNA gene functioned as a reference gene. The melting curve for the 16S rRNA gene is illustrated in (Figure 3A), revealing a single peak that signifies the specificity of the PCR product. The logarithmic phase of the 16S rRNA gene replication is shown in (Figure 3B).

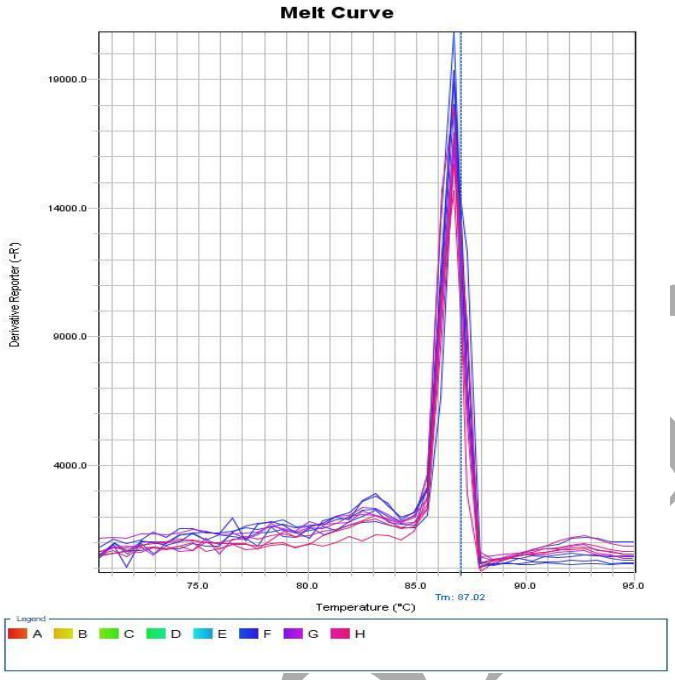


Figure 3A. Melting curve of 16S rRNA gene

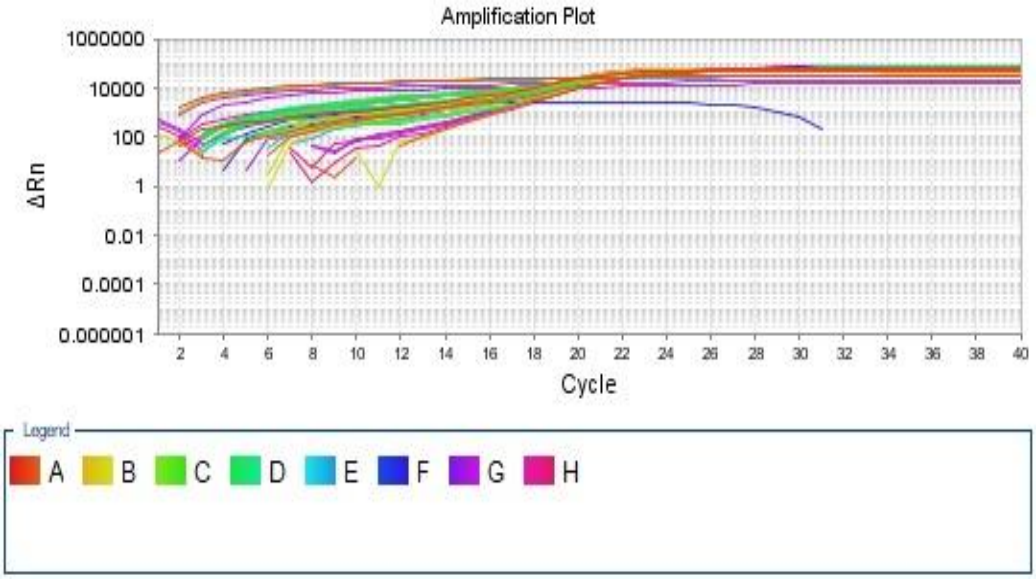


Figure 3B. 16srRNA gene amplification

3.5 Results of the Study on *tdh* Gene Expression Using the Cyber Green Real-Time PCR Technique

The expression of the *tdh* gene was quantified using Real-time PCR, with analyses conducted on the melting curve, standard curve, and amplification graph. The standard curve for the *tdh* gene, which assesses the efficiency of the Real-time PCR technique, is presented in (Figure 4). The melting curve of the *tdh* gene is shown in (Figure 5). Figure 6 illustrates the average variation in *tdh* gene expression in samples treated with probiotics in comparison to control samples.

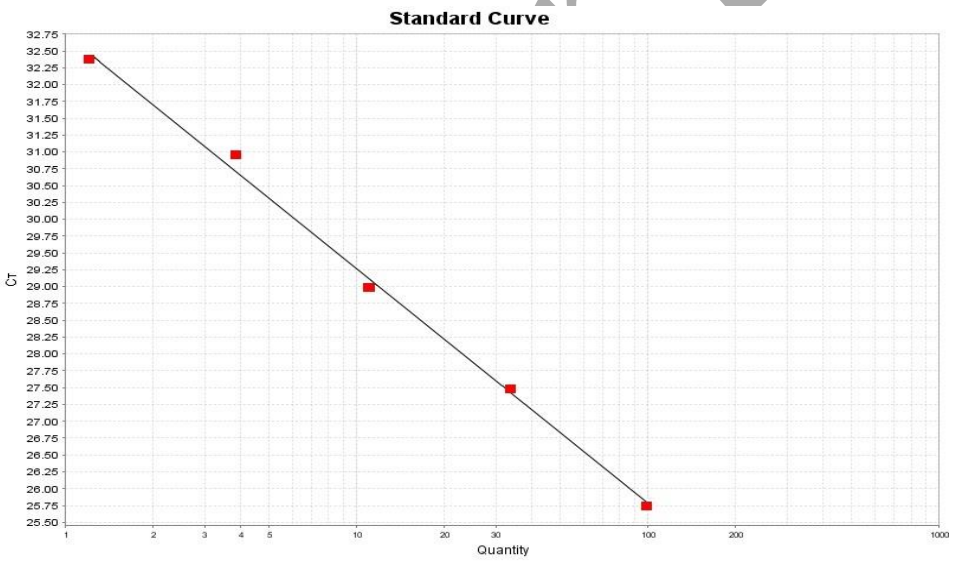
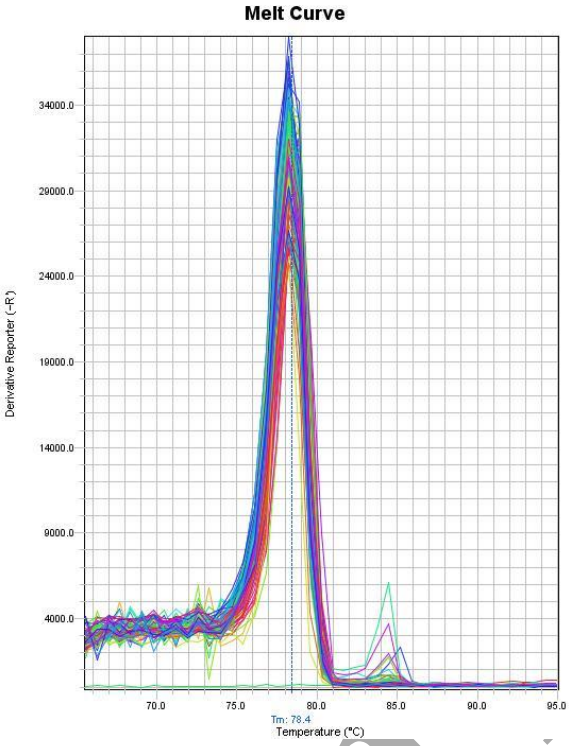


Figure 4. *tdh* gene standard curve



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296 Figure 5. Melting curve of the *tdh* gene

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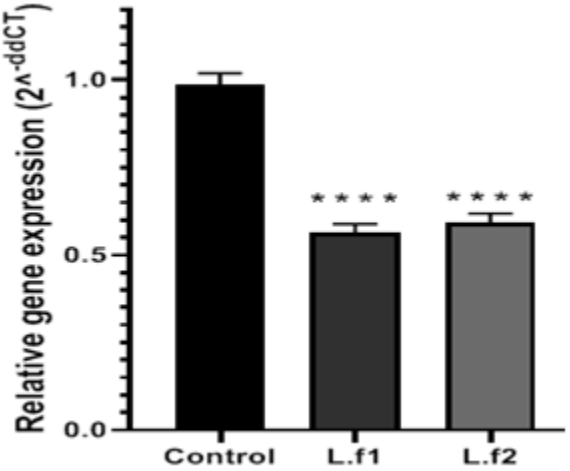

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300 Figure 6. The mean variation in *tdh* gene expression in probiotic-treated samples (L.f1: *L. fermentum* and
301 L.f2: *L. plantarum)* was assessed in comparison to control samples.

4. Discussion

The growing reliance on aquatic products as a major protein source has substantially increased the demand for farmed fish, which provide a stable and affordable supply [1]. Ensuring microbiological safety in aquaculture products is therefore of critical importance. In the present study, the prevalence of *V. parahaemolyticus* was investigated in two widely consumed farmed fish species (trout and carp) distributed in Tehran. Among 264 examined samples, 7.57% were positive for *V. parahaemolyticus*, indicating the presence of this pathogen in retail aquaculture products.

The observed prevalence is comparable to several national and international reports, although considerable geographical variability exists [11]. Similar contamination levels have been reported in subtropical regions (7.5% in fish), while markedly higher rates were documented in Malaysia (42.9% in shrimp) [12], Bangladesh (60% in aquaculture farms) [14], and Korean coastal waters (34%) [15]. A study conducted on the Caspian Sea also detected *V. parahaemolyticus* in 20% of environmental samples [13], and a review study reported prevalence rates of 14% and 35% in Greece and Portugal, respectively, with no detection in England and France [16]. These discrepancies may be attributed to differences in ecological conditions, seasonal variation, sampling sites, sample types, and hygienic practices during harvesting and distribution [13]. Given the high production and consumption of trout and carp, even moderate contamination levels may represent a relevant public health concern [13,14,15].

A study conducted in Italy reported a significantly higher prevalence of Vibrio species, particularly *V. parahaemolyticus*, at 7.6%, which markedly surpasses our findings [17]. Comparisons between our results and similar studies conducted in Iran and other countries suggest that variations in the prevalence of *V. parahaemolyticus* and other Vibrio species in aquatic animals may be influenced by factors such as the sampling site, the nature of the samples, the season of sampling, and the ecological and geographical conditions of the region, alongside considerable differences in sanitary practices during fishing and product supply [13]. The high production of farmed fish and the increasing consumption of these products by Iranian families underscore the necessity of ensuring their health and safety. The results of our study, in conjunction with findings from other research, emphasize the relevance of *V. parahaemolyticus*

presence in farmed fish, particularly in the two most commonly consumed species, trout and carp [13,14,15].

The detection of virulence-associated genes further highlights the potential pathogenicity of the isolates [1]. In this study, the *tdh* gene was detected in 80% of isolates, whereas *trh* was not identified. Previous studies have reported variable frequencies of these virulence genes. For instance, Gutierrez West et al. documented *tdh* and *trh* frequencies of approximately 48% and 8.3%, respectively [18]. Similarly, Yan et al. reported lower detection rates of *tdh* (5.2%) and *trh* (3.9%) among aquatic isolates [19], while Yang et al. observed prevalence rates of 16.8% for *tdh* and 24.1% for *trh*, particularly during warmer seasons [20]. Such variability may reflect regional ecological differences and strain heterogeneity. The presence of toxigenic strains in farmed fish underscores the potential risk of gastroenteritis, particularly when seafood is inadequately cooked [19].

The antimicrobial resistance patterns observed in this study are consistent with previous findings. High susceptibility to ciprofloxacin, chloramphenicol, and gentamicin, alongside significant resistance to ampicillin and tetracycline, has been widely reported [3,15,20]. Recent studies from South Korea documented ampicillin resistance rates as high as 58.8%, with all isolates harboring virulence-associated genes [21], while Yang et al. reported 79.5% resistance to ampicillin and 68.3% multidrug resistance [20]. These findings reflect the selective pressure imposed by the prolonged use of antibiotics in aquaculture systems and highlight the necessity of alternative control strategies.

In this context, probiotics have gained increasing attention as sustainable biocontrol agents. The present study demonstrated that culture supernatants of *L. fermentum* and *L. plantarum* exhibited significant inhibitory activity against *V. parahaemolyticus*. Supporting evidence indicates that probiotic supplementation can enhance growth performance and immune responses in aquaculture species [22]. For example, dietary administration of host-derived *L. plantarum* improved immune-related gene expression and disease resistance in rainbow trout [22]. Furthermore, probiotics have been shown to suppress pathogenic bacteria and enhance resistance against streptococcal infections in fish [23], and their role in controlling vibriosis has been

extensively reviewed [24]. Collectively, these findings support the incorporation of probiotic-based strategies as alternatives to antibiotics in aquaculture production.

Overall, the presence of toxigenic and partially antibiotic-resistant *V. parahaemolyticus* in farmed fish highlights ongoing public health concerns. Strengthening hygienic measures, continuous monitoring, rational antibiotic use, and the application of probiotic interventions may contribute to improving food safety and sustainability in aquaculture systems.

5. Conclusion

This study provides significant insights into the levels of microbial contamination present in farmed trout and carp sold across various regions of Tehran. The identification of toxigenic genes within these samples indicates that the consumption of raw or undercooked fish could lead to digestive complications, including diarrhea and gastroenteritis. Furthermore, the implementation of robust health monitoring protocols in fish farming and distribution centers has the potential to substantially reduce aquatic pollution. Probiotics are essential in enhancing aquatic environments and improving the existing microbiome. They can inhibit aquatic pathogens through diverse metabolites and mechanisms, and their incorporation as dietary supplements can significantly decrease dependence on antibiotics, thereby reducing overall antibiotic consumption.

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Authors' Contribution

MMSD, ZR: Conceptualization; MMSD, ZR: Methodology. EY, ZR: Investigation. MMSD, ZR, EY: Acquisition and analysis. HM: Writing the original draft. HM, MMSD: Writing the review and editing.

Ethics

This research was approved by the Ethics Committee of Tehran University of Medical Sciences under the code IR.TUMS.SPH.REC.1401.052

Funding

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Conflict of Interest

The authors declare that they have no competing interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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