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RESEARCH ARTICLE

Multiplex PCR assay for the accurate and rapid detection and differentiation of *Lactococcus garvieae* and *L. petauri*

Dilek Ustaoglu[1,2] | Rafet Çağrı Öztürk[1,2] | Mustafa Ture[3] | Silvia Colussi[4] | Paolo Pastorino[4] | Ana Isabel Vela[5] | Charalampos Kotzamanidis[6] | Donatella Volpatti[7] | Pier Luigi Acutis[4] | Ilhan Altinok[1,2,8]

[1]Department of Fisheries Technology Engineering, Faculty of Marine Sciences, Karadeniz Technical University, Trabzon, Turkey

[2]Aquatic Animal Health and Molecular Genetic Lab, Karadeniz Technical University, Trabzon, Turkey

[3]Department of Fish Health, Central Fisheries Research Institute, Trabzon, Turkey

[4]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy

[5]VISAVET and Department of Animal Health, Universidad Complutense de Madrid, Madrid, Spain

[6]Veterinary Research Institute, ELGO-DIMITRA, Thessaloniki, Greece

[7]Department of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, Udine, Italy

[8]Institute of Marine Sciences and Technology, Karadeniz Technical University, Trabzon, Turkey

Correspondence
Ilhan Altinok, Department of Fisheries Technology Engineering, Faculty of Marine Sciences, Karadeniz Technical University, Trabzon 61530, Turkey.
Email: ialtinok@gmail.com

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Abstract

Lactococcosis is a common bacterial fish disease caused by *Lactococcus garvieae*, *L. petauri and L. formosensis*. Although there are different PCR-based techniques to identify the etiological agent, none of these can differentiate these two bacteria without sequencing PCR-amplified fragments. In the present study, we developed a multiplex PCR assay for simultaneous detection and differentiation of *L. garvieae* and *L. petauri*. The specificity of the primers was validated against the bacterial DNA of the targeted and non-targeted bacteria. The sizes of the PCR amplicons were obtained as 204 bp for the DUF1430 domain-containing protein gene of *L. garvieae*, 465 bp for the Lichenan permease IIC component gene of *L. petauri*, and 302 bp for the teichoic acid biosynthesis protein F gene of both *L. garvieae* and *L. petauri*. The PCR amplicons were clearly separated by agarose gel electrophoresis. The multiplex PCR assay did not produce any amplification products with the DNA of the non-targeted bacteria. The multiplex PCR detection limits for *L. garvieae* and *L. petauri* were 5 and 4 CFU in pure culture and 50 and 40 CFU/g in spiked tissue samples, respectively. It takes less than 2 h from plate-cultured bacteria and 3 h from tissue samples to get results. In conclusion, the developed multiplex PCR assay is a rapid, specific, accurate, and cost-effective method for the detection and differentiation of *L. garvieae* and *L. petauri* and is suitable to be used for routine laboratory diagnosis of *L. garvieae* and *L. petauri*.

KEYWORDS
lactococcosis, *Lactococcus garvieae*, *Lactococcus petauri*, multiplex PCR, salmonids

1 | INTRODUCTION

Lactococcosis, caused by *Lactococcus garvieae*, *L. petauri* and *L. formosensis*, is a systemic hyperacute bacterial disease that affects a broad range of freshwater and marine species, including rainbow trout (Altinok et al., 2022), sea bass (Salogni et al., 2024), cobia (Rao et al., 2022), mullet (Tsai et al., 2012), tilapia (Bwalya et al., 2021), largemouth black bass (Abraham et al., 2023), shrimp (Ballantyne et al., 2023), octopus (Fichi et al., 2015), and prawn (Chen et al., 2001). Water temperature is one of the most important environmental factors in the development of lactococcosis outbreaks. While fish are asymptomatic at lower temperatures, the severity of the disease increases at water temperatures above 15°C and causes significant economic losses, especially in farmed rainbow trout (Castro et al., 2017; Khalil et al., 2023, 2024; Soltani et al., 2021).

Although *L. garvieae* has been recognized as the only causative agent of lactococcosis since its description in the early 1990s (Collins et al., 1983), two novel species, *L. petauri* (Goodman et al., 2017) and *L. formosensis* (Abraham et al., 2023), were linked with lactococcosis. In 2020, *L. petauri* was reported from a lactococcosis outbreak in rainbow trout (Kotzamanidis et al., 2020). Since then, *L. petauri* has been reported from Türkiye (Altinok et al., 2022), the USA (Abraham et al., 2023; Littman et al., 2023), Brazil (Egger et al., 2023), Greece, and Spain (Stoppani et al., 2023).

Different strains and species of bacteria have different levels of virulence and pathogenicity. Accurate, fast and feasible identification/discrimination of bacterial species or strains is crucial for disease monitoring, understanding transmission dynamics, and implementing appropriate and rapid control measures. Due to the phenotypic and genetic similarities between *L. garvieae* and *L. petauri*, traditional microbial characterization techniques such as MALDI-TOF and PCR assays that target a small portion of the genome do not accurately discriminate at the species level (Egger et al., 2023; Martinovic et al., 2021). Some *L. petauri* isolates were misidentified as *L. garvieae* based on phenotypic methods and gene sequencing (Altinok et al., 2022; Egger et al., 2023; Goodman et al., 2017; Kotzamanidis et al., 2020). For instance, based on phenotypic characteristics and 16S rRNA gene sequences, Ture (2011) characterized a pathogen isolated from a lactococcosis outbreak in farmed rainbow trout in Türkiye as lg-per strain and identified the strain as *L. garvieae*. Thereafter, 16S rRNA gene sequences were used in the following studies for *L. garvieae* species confirmation (Ture et al., 2018; Ture & Altinok, 2016). Whole genome sequencing (WGS) confirmed that the Lg-per strain was, in fact, *L. petauri* (Altinok et al., 2022). It was also found that the sequence analysis of 16S rRNA gene of *L. garvieae* was not suitable for species identification due to the 99.9% similarity between *L. garvieae* and *L. petauri*. Similarly, Vela et al. (2024) assessed the species assignment of bacteria isolated from rainbow trout farms across Europe, which were initially identified as *L. garvieae*, by comparing whole genome sequences and revealed that 29 out of 48 isolates that were previously identified as *L. garvieae* were indeed *L. petauri*. The calculation of average nucleotide identity values between whole genome sequences is considered an accurate method for identifying prokaryotes (Arahal, 2014). Yet it is labor-intensive, time-consuming, and costly.

Several PCR assays have been developed for the rapid identification and discrimination of *L. garvieae* and *L. petauri*. Stoppani et al. (2023) distinguished *L. garvieae* and *L. petauri* based on five diagnostic variable sites in the internal transcribed spacer region between 16S rRNA and 23S rRNA. While *L. garvieae* and *L. petauri* can be accurately differentiated based on 16S-23S ITS gene variation, it requires a gene sequencing step, which can delay the diagnosis and increases cost. Saticioglu et al. (2023) designed a multiplex PCR assay using two different primer pairs to target the Glycosyltransferase family 4 protein coding gene of *L. garvieae* and the ABC transporter permease (TagG) of *L. petauri* for the detection and differentiation of *L. petauri* and *L. garvieae*. During the SUPERTROUT European Project (Partnership for Research and Innovation in the Mediterranean Area, 2019), we screened the *Lactococcus* spp. isolates using primer sets from Saticioglu et al. (2023). Our findings indicate that certain *L. petauri* strains, confirmed by whole genome sequencing, were amplified by *L. garvieae* primers. Therefore, it appears that the suggested LG_IBS primers lack specificity and cross-react with some strains of *L. petauri*. Considering the importance of rapid diagnostic tests for differentiating *L. garvieae* and *L. petauri*, an accurate and cost-effective method is still needed for efficient control of lactococcosis outbreaks. In the present study, we developed a novel rapid and accurate multiplex PCR assay for distinguishing *L. garvieae* and *L. petauri* from natural outbreaks, pure cultures, and spiked samples.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates

In the present study, three reference strains of *Lactococcus garvieae* and 116 *Lactococcus* spp. isolated from outbreaks of lactococcosis in rainbow trout farms in Türkiye, Italy, Greece, and Spain were used. In a previous study, these isolates were identified at species levels by using 16S–23S rRNA internal transcription spacer region sequencing, as described by Stoppani et al. (2023). Additionally, 10 of these isolates were identified at the species level via whole genome sequencing (please see Altinok et al., 2022; Vela et al., 2024) and used as controls (Table 1).

2.2 | Primer design

Three primer sets (Table 2) were designed to identify the specific genes that encode the DUF1430 domain-containing protein found exclusively in *L. garvieae* and the Lichenan permease IIC component found exclusively in *L. petauri*. Only two of the 50 primer sets used for primer selection were found to be unique to *L. garvieae* and

TABLE 1 Details of the *Lactococcus* spp. Isolates used in the present study.

Isolate/strain	Year of isolation	Country	Identification based on ITS sequencing	Identification based on WGS	Sucrose fermentation	Whole genome accession number
Y-LG1	2016	Türkiye	*L. petauri*	*L. petauri*	+	JAQPON000000000
LG10	2017	Türkiye	*L. petauri*	*L. petauri*	+	JAQPOM000000000
LG6	2008	Greece	*L. petauri*	*L. petauri*	+	JAOYNZ010000000
LG5	2009	Greece	*L. petauri*	*L. petauri*	+	JAOYNY010000000
LG3	2010	Greece	*L. petauri*	*L. petauri*	+	JAOYNX010000000
LG1	2016	Greece	*L. petauri*	*L. petauri*	+	JAOYNW010000000
6071	2002	Spain	*L. petauri*	*L. petauri*	+	JARHWN000000000
8568	2003	Spain	*L. petauri*	*L. petauri*	+	JARHWM000000000
8864	2004	Spain	*L. petauri*	*L. petauri*	+	JARHWK000000000
935	2016	Spain	*L. petauri*	*L. petauri*	+	JARHWC000000000
DSM 104842	2017	Reference strain	*L. petauri*		+	
DSM 20684	1990	Reference strain	*L. garvieae*		–	
DSM 6783	1991	Reference strain	*L. garvieae*		–	
YLG2	2016	Türkiye	*L. petauri*		+	
YLG3	2016	Türkiye	*L. petauri*		+	
YLG6	2016	Türkiye	*L. petauri*		+	
YLG7	2017	Türkiye	*L. petauri*		+	
KLG3	2016	Türkiye	*L. petauri*		+	
KLG7	2018	Türkiye	*L. petauri*		+	
KLG8	2018	Türkiye	*L. petauri*		+	
KURTUN2020	2020	Türkiye	*L. petauri*		+	
RIZE2020	2020	Türkiye	*L. petauri*		+	
VAKFIKEBIR	2020	Türkiye	*L. petauri*		+	
LG10	2017	Türkiye	*L. petauri*		+	
LG13	2016	Türkiye	*L. petauri*		+	
LG20	2016	Türkiye	*L. petauri*		+	
LG107B	2017	Türkiye	*L. petauri*		+	
LG123	2016	Türkiye	*L. petauri*		+	
LG140	2019	Türkiye	*L. petauri*		+	
TR71	2022	Türkiye	*L. petauri*		+	
TR72	2022	Türkiye	*L. petauri*		+	
TR73	2022	Türkiye	*L. petauri*		+	
TR74	2022	Türkiye	*L. petauri*		+	
TR75	2022	Türkiye	*L. petauri*		+	
TR76	2022	Türkiye	*L. petauri*		+	
TR77	2022	Türkiye	*L. petauri*		+	
TR78	2022	Türkiye	*L. petauri*		+	
TR79	2022	Türkiye	*L. petauri*		+	
TR81	2022	Türkiye	*L. petauri*		+	
TR82	2022	Türkiye	*L. petauri*		+	
TR83	2022	Türkiye	*L. petauri*		+	
TR84	2022	Türkiye	*L. petauri*		+	
TR85	2022	Türkiye	*L. petauri*		+	
TR86	2022	Türkiye	*L. petauri*		+	
GR_Lg 1	2016	Greece	*L. petauri*		+	

(Continues)

TABLE 1 (Continued)

Isolate/strain	Year of isolation	Country	Identification based on ITS sequencing	Identification based on WGS	Sucrose fermentation	Whole genome accession number
GR_Lg 2	2010	Greece	*L. petauri*		+	
GR_Lg 3	2010	Greece	*L. petauri*		+	
GR_Lg 4	2010	Greece	*L. petauri*		+	
GR_Lg 5	2009	Greece	*L. petauri*		+	
GR_Lg 6	2008	Greece	*L. petauri*		+	
GR_Lg 7	2009	Greece	*L. petauri*		+	
GR_Lg 8	2009	Greece	*L. petauri*		+	
GR_Lg 10	2009	Greece	*L. petauri*		+	
GR_Lg 11	2007	Greece	*L. petauri*		+	
GR_Lg 12	2010	Greece	*L. petauri*		+	
GR_Lg 13	2010	Greece	*L. petauri*		+	
GR_Lg 15	2008	Greece	*L. petauri*		+	
GR_Lg 16	2006	Greece	*L. petauri*		+	
GR_Lg 17	2006	Greece	*L. petauri*		+	
GR_Lg 18	2007	Greece	*L. petauri*		+	
GR_Lg 19	2008	Greece	*L. petauri*		+	
GR_Lg 20	2007	Greece	*L. petauri*		+	
GRLP30	2022	Greece	*L. petauri*		+	
GRLP31	2022	Greece	*L. petauri*		+	
GRLP32	2022	Greece	*L. petauri*		+	
GRLP33	2022	Greece	*L. petauri*		+	
29465/IB	2016	Italy	*L. garvieae*		−	
29487/1B	2016	Italy	*L. garvieae*		−	
27297/1	2016	Italy	*L. garvieae*		−	
27297/2	2016	Italy	*L. garvieae*		−	
65166/1B	2017	Italy	*L. garvieae*		−	
65186/1B	2017	Italy	*L. garvieae*		−	
76032/1	2018	Italy	*L. garvieae*		−	
86771/3	2018	Italy	*L. garvieae*		−	
86761/1	2018	Italy	*L. garvieae*		−	
40345/1	2019	Italy	*L. garvieae*		−	
41228/1	2019	Italy	*L. garvieae*		−	
58503/1	2019	Italy	*L. garvieae*		−	
58502/1	2019	Italy	*L. garvieae*		−	
88709	2020	Italy	*L. garvieae*		−	
88710	2020	Italy	*L. garvieae*		−	
25865/4	2017	Italy	*L. garvieae*		−	
28050/2	2017	Italy	*L. garvieae*		−	
41228/5	2019	Italy	*L. garvieae*		−	
58507/2	2019	Italy	*L. garvieae*		−	
40345/2	2019	Italy	*L. garvieae*		−	
77414/1	2022	Italy	*L. garvieae*		−	
77413/4	2022	Italy	*L. garvieae*		−	
77404/5	2022	Italy	*L. garvieae*		−	
77991/2	2022	Italy	*L. garvieae*		−	

TABLE 1 (Continued)

Isolate/strain	Year of isolation	Country	Identification based on ITS sequencing	Identification based on WGS	Sucrose fermentation	Whole genome accession number
77391/2	2022	Italy	*L. garvieae*		–	
77404/2	2022	Italy	*L. garvieae*		–	
77414/7	2022	Italy	*L. garvieae*		–	
77987/7	2022	Italy	*L. garvieae*		–	
77977/4	2022	Italy	*L. garvieae*		–	
77409/3	2022	Italy	*L. petauri*		+	
820	2016	Spain	*L. petauri*		+	
8568	2003	Spain	*L. petauri*		+	
6071	2002	Spain	*L. petauri*		+	
5664	2017	Spain	*L. petauri*		+	
8666	2016	Spain	*L. petauri*		+	
8943	2016	Spain	*L. petauri*		+	
5787	2016	Spain	*L. petauri*		+	
8831	2017	Spain	*L. petauri*		+	
8495	2016	Spain	*L. petauri*		+	
195	2019	Spain	*L. petauri*		+	
8516	2016	Spain	*L. petauri*		+	
1008	2017	Spain	*L. petauri*		+	
5239	2019	Spain	*L. petauri*		+	
393	2019	Spain	*L. petauri*		+	
818	2016	Spain	*L. petauri*		+	
8059	2016	Spain	*L. petauri*		+	
307	2016	Spain	*L. petauri*		+	
8864	2004	Spain	*L. petauri*		+	
935	2016	Spain	*L. petauri*		+	
5424	2017	Spain	*L. petauri*		+	
SAP22/01792	2022	Spain	*L. petauri*		+	
SAP22/01795	2022	Spain	*L. petauri*		+	
SAP22/01796	2022	Spain	*L. petauri*		+	

TABLE 2 Details of primers developed in this study, size of PCR amplicons, targeted genes.

Primer pair	Sequences	Amplicon size (bp)	Target gene	GenBank Acc.
LG211F	GTTGACGAGCAGAACACCTT	204	DUF1430 domain-containing protein	AP009333
LG414R	TCGAGGCACACAGAGATGTT			
LP304F	GGCGTTCTCTCATTAGCAGC	465	Lichenan permease IIC component	CP094882
LP768R	CATCGGACGAACAATGGAGC			
ICTabBF	GGATGTGCTATCCGTTGCCT	302	Teichoic acid biosynthesis protein F	AP009333 CP094882
ICTabBR	TCTACAAGGCGCATCCACTT			

L. petauri. Other primer sets exhibited cross-reactivity. The primer pair designed to amplify the gene encoding the teichoic acid biosynthesis protein F serves as an internal control for both *L. garvieae* and *L. petauri*. The primers were designed, and their specificity was evaluated using the Primer Blast pipeline program (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

2.3 | DNA extraction from pure culture

Isolates were grown on TSA agar and incubated overnight at 37°C. A sucrose fermentation test suggested by Vela et al. (2024) as a diagnosis tool for the etiological agent of lactococcosis was performed on all isolates. The genomic DNA of the isolates was extracted with the

NucleoGene DNA extraction buffer following the manufacturer's instructions (NucleoGene, Istanbul, Türkiye). A single colony was transferred to a 0.5 mL centrifuge tube containing 100 μL of one-step DNA extraction buffer. Following 15 min of incubation at 95°C in a thermal cycler, samples were placed at −20°C for 10 min. Following thawing, samples were gently vortexed, centrifuged at 10,000*g* for 5 min, and the supernatant containing DNA was transferred to another centrifuge tube and kept at −20°C until analysis. A spectrophotometer (Nano-200, Allsheng Inc., China) was used to measure DNA concentration and purity. Using a low TE buffer, 10-fold dilutions of the extracted DNA were made at concentrations ranging from 100 ng/μL to 0.1 pg/μL.

2.4 | Multiplex PCR assay and sanger sequencing

PCR was carried out in a total volume of 20 μL, containing 4 μL of 5× Hot Start Multiplex PCR Master mix (NucleoGene, Istanbul, Türkiye), 1 μL of each primer mix [LG211-414 (1 μM), LP304-768 (1 μM), ICTabB (1 μM)], 2 μL of template DNA, and dH2O. A PCR reaction mixture without template DNA was used as a negative control. PCR mixtures containing *L. garvieae* (ATCC 49156) and *L. petauri* (Lg-per) were used as positive controls. The thermal cycling conditions were as follows: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were visualized on 1.5% agarose gel. Each fragment was extracted from the gel using QIAquick Gel extraction kit (Qiagen) following the manufacturer's protocol and bidirectionally sequenced on an ABI 3500 Genetic Analyser (Applied Biosystems, USA) with the BigDye Terminator Cycle Sequencing Kit v.3.1. The raw reads were merged, edited, and aligned in BioEdit (Hall, 1999). Sequences were compared with available sequences in the GenBank database (NCBI) using basic local alignment search tool (BLAST) and deposited in GenBank. The PCR products were confirmed to be from the *L. garvieae* DUF1430 domain-containing protein gene (GenBank accession: PP693217), the *L. garvieae* teichoic acid biosynthesis protein F gene (GenBank accession: PP693218), and the *L. petauri* lichenan permease IIC component gene (GenBank accession: PP693219).

2.5 | PCR specificity and sensitivity

The specificity of the multiplex PCR assay was tested with 116 *Lactococcus* spp. and non-targeted bacteria including *L. lactis* (IT20729 and IT20175), *L. piscium* (DSMZ 6634), *Streptococcus iniae* (DSMZ 20576), *S. agalactiae* (DSMZ 16828), *S. dysgalactiae* (DSMZ 20662), *S. parauberis* (DSMZ 6631), *Carnobacterium maltaromaticum* (IT33565), *C. divergens* (IT33604), *Enterococcus faecalis* (V583), *E. gallinarum* (BM4174), *Aeromonas hydrophila* (ATCC 7966), *Escherichia coli* (JM107), Yersinia ruckeri (TSE075), and *Vibrio anguillarum* (LMG10861).

The sensitivity and detection limits of the multiplex PCR assay were tested using pure bacterial cultures and tissue spiked in vitro with bacterial cultures. *L. garvieae*, ATCC 49156 strain, and *L. petauri*, lg-per strain, were used. Bacteria, grown in BHI broth for 24 h at 37°C, were serially diluted in PBS from 10^8 to 10^1 CFU/mL according to Altinok (2011). Each dilution was spread on TSA to determine colony forming units. DNA was extracted from pelleted cells from each dilution following the same DNA extraction method given above and used as template DNA in a multiplex PCR assay following the same PCR conditions given above. These experiments were done in triplicate.

The sensitivity of the multiplex PCR assay in the presence of fish tissue was performed on rainbow trout kidney spiked *in vitro* with *L. garvieae* ATCC 49156 and *L. petauri* lg-per. A total of 100 mg of the rainbow trout kidney was homogenized by using a homogenizer (Bioprep-24, Allsheng, China) and seeded with serial dilutions of an equal mixture of bacteria as described in Altinok (2011). Each dilution was spread on TSA to determine the colony-forming units. DNA was extracted with the classic phenol-chloroform isolation method, according to Ausubel et al. (1988). A multiplex PCR assay was performed as described above.

2.6 | Diagnosis of lactococcosis from a natural outbreak

During a natural disease outbreak in the summer of 2021 and 2022, when the water temperature hovered around 18°C, rainbow trout samples showing gross clinical signs of lactococcosis were collected from a cage system located on a dam. A total of 92 fish were sampled for bacterial examination, and swab samples were collected from the oral cavities of all fish using special swabs (4 N6 FLOQSwabs, Copan, Italy) containing an active drying system that absorbs water. Fish were anaesthetized with benzocaine (25 mg/L) during sampling. The swab samples were maintained at room temperature for a period of 1 to 2 years. Subsequently, DNA was extracted from the swab with the Nucleospin Tissue XS (Macherey-Nagel, Germany) following the manufacturer's protocol for buccal swabs, and a multiplex PCR test was performed to assess the presence of *L. garvieae* and *L. petauri*, as previously described.

3 | RESULTS

3.1 | PCR amplification

Before designing our new PCR primers to differentiate between closely related *L. garvieae* and *L. petauri*, we first used the primers developed by Saticioglu et al. (2023) to identify *L. garvieae* and *L. petauri isolates/strains* from various countries. This was done as

part of our ongoing PRIMA project. The LG_IBS primers targeting the Glycosyltransferase family 4 protein-coding gene of *L. garvieae* cross-reacted with *L. petauri* strains and resulted in the production of 600-bp fragments (Figure 1.). These *L. petauri* strains were previously confirmed by whole genome sequencing.

3.2 | Optimization of the multiplex PCR assay

DNA extracted from 30 isolates of *L. garvieae*, 86 isolates of *L. petauri*, and 15 isolates of other non-targeted species were used in a multiplex PCR assay. The optimum annealing temperature for the multiplex PCR assay was found to be 55°C. The developed multiplex PCR assay accurately amplified the targeted DUF1430 domain-containing protein gene of *L. garvieae* at 204 bp, Lichenan permease IIC component of *L. petauri* at 465 bp, and teichoic acid biosynthesis protein F of both *L. garvieae* and *L. petauri* at 302 bp (Figure 2). The assembled sequences of amplicons were compared with reference sequences in the GenBank database by BLAST analysis, which revealed 99%–100% homology, indicating high specificity of the multiplex PCR assay.

3.3 | Specificity and sensitivity of the multiplex PCR assay and sucrose fermentation test

The specificity of the multiplex PCR assay was verified by using the DNA of related but non-targeted bacteria (*L. lactis* and *L. piscium*) and unrelated bacteria (*S. parauberis*, *S. dysgalactiae*, *S. iniae*, *S. agalactiae*, *C. maltaromaticum*, *C. divergens*, *V. anguillarum*, *Y. ruckeri*, and *A. hydrophila*). In all cases, there was no PCR amplification in the DNA of non-targeted and unrelated bacteria, indicating that the multiplex PCR assay does not cross-react with other species. In other words, amplification was observed only in the presence of *L. garvieae* and *L. petauri* DNA, which indicates the specificity of the primers. All the *L. garvieae* isolates were negative for sucrose utilization, whereas all the *L. petauri* isolates were positive for sucrose utilization.

The minimum detection limit in the multiplex PCR was 5 CFU for *L. garvieae* and 4 CFU for *L. petauri* when pure cultures were used. The detection limits for the spike samples were 40 CFU/g of tissue for *L. garvieae* and 30 CFU/g of tissue for *L. petauri* (Figure 3).

3.4 | Diagnosis of lactococcosis from a natural outbreak

Following the examination of one or two-year-old buccal swabs stored at room temperature, a multiplex PCR analysis revealed the existence of two distinct bands. One band indicated the presence of *L. petauri*, while the other band served as an internal control. Swap samples collected from all fish ($n = 95$) were found to be *L. petauri* positive. Similarly, *L. petauri* was isolated from the kidney and spleen of all these fish. Hence, *L. garvieae* was not detected.

4 | DISCUSSION

Multiplex PCR assays are efficient in clinical diagnosis and have been widely applied to detect and differentiate pathogenic bacteria based on amplifying the unique genes of interest (Altinok, 2011; Altinok et al., 2008; Kang et al., 2023; Wei et al., 2013). A novel and rapid multiplex PCR protocol was developed to simultaneously detect and distinguish *L. garvieae* and *L. petauri*. *L. petauri* and *L. garvieae* are emerging fish pathogens, causing significant losses in cultured salmonids at elevated water temperatures. Fast and accurate identification and discrimination of these pathogens are important for effective treatment. While 16S rRNA gene sequencing was once considered the gold standard for microbial identification, its resolution power is not high enough to differentiate closely related species that share high sequence similarity (García-López et al., 2014; Lan et al., 2016; Ture & Boran, 2015). Whole genome sequencing, on the other hand, holds the potential to identify and differentiate pathogens more precisely, and it is becoming a widely used technique in clinical diagnostics (Gautam et al., 2019).

Based on our current knowledge, there are only two studies on the simultaneous detection and differentiation of *L. garvieae* and *L. petauri* (Saticioglu et al., 2023; Stoppani et al., 2023). We assessed the accuracy of the designed primers in these studies for detecting and differentiating *L. garvieae* and *L. petauri*. Primers designed by Stoppani et al. (2023) showed high specificity and sensitivity, yet identification relies on amplicon sequencing, which can delay the diagnosis and increase the cost. On the other hand, a primer pair (LG_IBS), targeting the Glycosyltransferase family four protein-coding gene of *L. garvieae* in the multiplex PCR assay designed by Saticioglu et al. (2023), cross-reacted with *L. petauri* strains, which

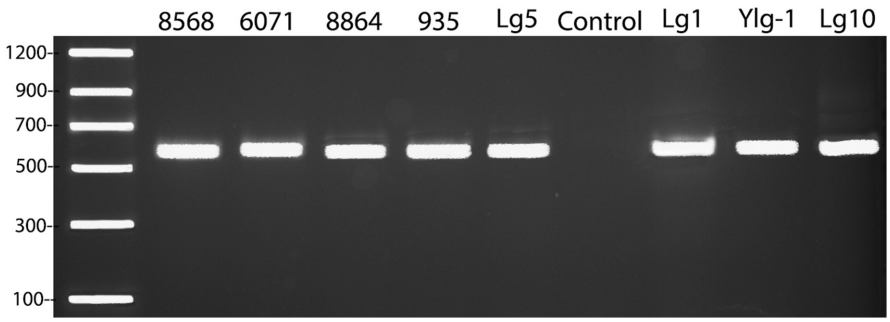

FIGURE 1 Cross-reaction of LG_IBS primers, designed by Satici et al. (2023), with *Lactococcus petauri* strains, of which species status were previously confirmed with whole genome sequence analysis (Whole genome sequences of these strains are available in GenBank, please see Table 1 for details).

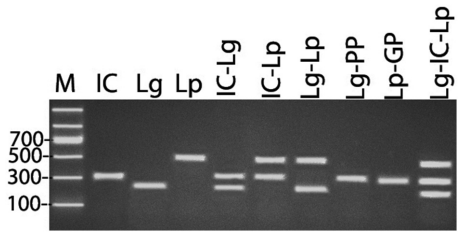

FIGURE 2 Specificity of the mPCR assay developed for the detection of *Lactococcus garvieae* and *Lactococcus petauri*. Lane M: DNA Marker II (Bostonchem) molecular size marker; Lane IC: Internal control (IC) containing IC primer and *L. garvieae* and *L. petauri* DNA (302 bp); Lane Lg: *L. garvieae* DNA and *L. garvieae* primers (204 bp); Lane Lp: *L. petauri* DNA and *L. petauri* primers (465 bp); Lane IC-Lg: IC and *L. garvieae* primers and *L. garvieae* DNA; Lane IC-Lp: IC and *L. petauri* primers and *L. petauri* DNA; Lane Lg-Lp: *L. garvieae* and *L. petauri* primers and *L. garvieae* and *L. petauri* DNA; Lane Lg-PP: IC and *L. petauri* primer and *L. garvieae* DNA; Lane Lg-GP: IC and *L. garvieae* primer and *L. petauri* DNA; Lane Lg-IC-Lp: IC, *L. garvieae* and *L. petauri* primers and *L. garvieae* and *L. petauri* DNA.

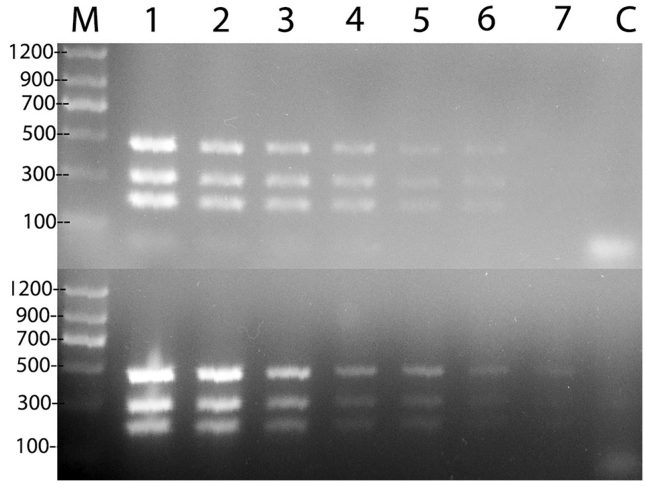

FIGURE 3 PCR amplification on tissue samples seeded with serially diluted cultures of *Lactococcus garvieae* and *L. petauri* (a). Sensitivity of the detection of *L. garvieae* and *L. petauri* by multiplex PCR assay from a pure culture (b). The PCR product sizes obtained for *L. garvieae*, *L. petauri*, and the internal control were 204, 465, and 302 bp, respectively. Lane M: DNA Marker size marker; Lanes 1 to 7: 10^{-1} to 10^{-7} dilution, respectively. For *L. garvieae* and *L. petauri*, the lowest dilution corresponds to 5 and 4 CFU in pure culture and 50 and 40 CFU/g in spiked tissue samples, respectively.

were previously identified based on whole genome sequencing at species level, and produced a false positive result, which indicates low specificity. Though the primers targeted conserved gene regions, variation within targeted bacteria might not be considered during primer designing process. This generally happens when primers are developed using sequences from a limited number of strains, or strains isolated from close proximities, rather than screening sequences of different isolates/strains available. The rates of false negative and false positive results also vary when the number of isolates in a laboratory differs from the available sequence data.

In this study, a total of 116 *L. garvieae* and *L. petauri* isolates/strains that were isolated in the last 10 years from lactococcosis outbreaks in trout farms in Türkiye, Italy, Spain, and Greece, along with 15 non-targeted related and unrelated bacteria, were used for testing the simultaneous detection and differentiation success of the multiplex PCR assay. The multiplex PCR assay successfully identified and differentiated all tested *L. garvieae* and *L. petauri* strains/isolates without producing any false negatives or false positives. The specificity and sensitivity of the multiplex PCR assay were high. The multiplex PCR assay did not cross-react with related non-targeted bacteria such as *L. lactis* and *L. piscium* or non-related non-targeted bacteria. In terms of sensitivity, the detection limit was as low as 3 CFU from pure culture and 30 CFU/g tissue from spiked tissues, suggesting that the multiplex PCR assay is sensitive enough to be applied for clinical diagnosis. The sensitivity and specificity results of the multiplex PCR assay were similar to those of multiplex PCR assays previously designed for the identification and differentiation of different bacteria (Altinok, 2011; Altinok et al., 2008; Zhang et al., 2014).

Routine screening of fish for diseases is important for the early detection and prevention of outbreaks, and more effective disease control and management strategies can be implemented. Traditional, standardized, and proven methods for bacterial examination require lethal sampling and are usually based on bacterial isolation in a culture medium. The use of non-lethal sampling techniques in microbiologic and molecular diagnostic tests is a promising approach for pathogen detection. In the present study, swap samples collected from moribund fish, showing clinical signs of lactococcosis, were found to be *L. petauri* positive. Identification of pathogens takes less than 3 h in the lab once the samples arrive. Yet, more studies are needed to validate the sensitivity and specificity of the method using asymptomatic fish. *L. garvieae* and *L. petauri* isolates used in this study were strictly distinguished by sucrose fermentation test proposed by Saticioglu et al. (2023), which confirms reliability of using sucrose fermentation test for differentiating *L. garvieae* and *L. petauri*. Still, it requires bacterial isolation and identification, at least at the genus level. Still, it is useful for quick identification and differentiation of available *Lactococcus* spp. collections. In cases, when fish exibit clinical symptoms, non-lethal sampling with swaps and PCR based differentiation protocol proposed in this study would be efficient for detecting of Lactococcosis disease and differentiation of *L. garvieae* and *L. petauri*.

5 | CONCLUSION

Compared to traditional detection methods, the availability of a multiplex PCR assay for rapid, specific, and sensitive diagnosis of closely related bacterial pathogens is important for effective treatment. The novel multiplex PCR assay developed in this study is highly sensitive and specific, and it may be a valuable tool for more rapid diagnosis and routine screening of fish tissue and pure bacterial cultures for *L. garvieae* and *L. petauri*. In a single reaction and in a single tube,

L. garvieae and *L. petauri* can be simultaneously detected and identified without relying on sequencing data. In addition, non-lethal sampling, collected from the oral cavity of a fish using swabs, is an alternative method for the diagnosis of *L. garvieae* and *L. petauri* in both diseased and carrier fish.

AUTHOR CONTRIBUTIONS

Dilek Ustaoglu: Investigation; writing – original draft. **Rafet Çağrı Öztürk:** Investigation; writing – original draft; writing – review and editing. **Mustafa Ture:** Investigation. **Silvia Colussi:** Investigation; project administration; funding acquisition. **Paolo Pastorino:** Investigation. **Ana Isabel Vela:** Investigation. **Charalampos Kotzamanidis:** Investigation. **Donatella Volpatti:** Investigation. **Pier Luigi Acutis:** Investigation. **Ilhan Altinok:** Conceptualization; supervision; methodology; funding acquisition; investigation; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

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

ORCID

Dilek Ustaoglu https://orcid.org/0000-0002-6947-3741
Rafet Çağrı Öztürk https://orcid.org/0000-0003-1785-4056
Mustafa Ture https://orcid.org/0000-0001-9878-826X
Silvia Colussi https://orcid.org/0000-0002-3261-4539
Paolo Pastorino https://orcid.org/0000-0002-0585-1168
Ana Isabel Vela https://orcid.org/0000-0001-5004-3103
Charalampos Kotzamanidis https://orcid.org/0000-0002-8804-239X
Donatella Volpatti https://orcid.org/0000-0002-6300-1735
Pier Luigi Acutis https://orcid.org/0000-0003-4814-2924
Ilhan Altinok https://orcid.org/0000-0003-3475-521X

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