Journal of Fish Diseases

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Journal of Fish Diseases

RESEARCH ARTICLE OPEN ACCESS

First Detection of *Lactococcus formosensis* subsp. *formosensis* in Rainbow Trout (*Oncorhynchus mykiss*) in Europe

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Received: 14 July 2025 | **Revised:** 25 September 2025 | **Accepted:** 29 September 2025

Funding: This study was supported by the Italian Ministry of Agriculture, Food Sovereignty and Forests through the supply chain programme RESILTROUT-6CFP code 23E03. The project, titled "RESILTROUT–Resilient Aquaculture to Global Changes: Research Supporting the Italian Trout Farming Sector", was approved under MASAF Decree No. 399082 of 28 July 2023 (CUP: J18H23000800007).

Keywords: aquatic microbiology | bacterial pathogen | fish health | freshwater fish | piscine lactococcosis

ABSTRACT

Lactococcus garvieae, *Lactococcus petauri* and *Lactococcus formosensis* are etiological agents of piscine lactococcosis, a disease reported in Italy since the early 1990s and linked to significant aquaculture losses. To the best of our knowledge, this study reports the first detection of *L. formosensis* subsp. *formosensis* in farmed rainbow trout (*Oncorhynchus mykiss*) in Europe. In 2024, a total of 70 trout were sampled in the Piedmont region (Northern Italy), as part of a health monitoring programme. Bacteriological and molecular analyses showed the presence of *L. garvieae* in four fish (5.7%) and the identification of *L. formosensis* subsp. *formosensis* in one single fish exhibiting mild clinical signs via 16S-23S rRNA ITS sequencing. Phylogenetic analysis confirmed the clustering of the isolate within the *L. formosensis* clade, clearly distinct from *L. garvieae* and *L. petauri*. Virulotype comparison showed greater similarity between *L. petauri* and *L. formosensis* subsp. *formosensis*, while the latter exhibited a distinct biochemical profile and was highly susceptible to antibiotics commonly used in aquaculture. Nevertheless, monitoring and advanced diagnostics are essential to clarify the role of this bacterium and to develop any necessary preventive and control measures.

1 | Introduction

Aquaculture, which surpassed capture fisheries in 2022 with 131 million tonnes, provides over 20% of animal protein for 3.2 billion people, with salmonids accounting for 6.9% of production (FAO 2024). Its growth, however, is increasingly limited by infectious diseases, intensified farming and climate change, which promote the emergence of conditions such as piscine lactococcosis (Lafferty et al. 2015; Rodger 2016).

Piscine lactococcosis is a bacterial disease characterised by hyper-acute or acute haemorrhagic septicemia in fish, leading to morbidity and mortality rates ranging from 20% to 50% (Avci et al. 2014; Kotzamanidis et al. 2020; Shahin et al. 2021). The first documented occurrence was reported in the 1970s in Japanese amberjack (*Seriola quinqueradiata*) (Kusuda and Salati 1993), followed by the initial outbreak in Europe affecting rainbow trout (*Oncorhynchus mykiss*) in 1998 (Eldar and Ghittino 1999). Since then, piscine lactococcosis has emerged as a significant

disease impacting both freshwater and marine aquaculture industries in Asia, Europe, Australia, South and North American continents, Africa and the Middle East (Eldar and Ghittino 1999; Evans et al. 2009; Meyburgh et al. 2017; Shahin et al. 2021, 2025; Mahmoud et al. 2023; Heckman et al. 2024). Disease control remains challenging due to the persistence of the bacterium in carrier fish, wildlife and environmental biofilms, as well as the limited effectiveness of antimicrobial treatments and vaccines (Shahin et al. 2021; Heckman et al. 2024). Management is further complicated by the long-standing assumption that the disease was caused solely by *Lactococcus garvieae*, overlooking the involvement of other emerging species (Heckman et al. 2024; Shahin et al. 2025).

Lactococcus formosensis and *Lactococcus petauri* are two recently identified bacterial species that are closely related to *L. garvieae* (Chan et al. 2024). It is currently unclear whether these three species differ in host preference, geographical range, or accessory genes, including virulence and antimicrobial resistance genes (Chan et al. 2024; Heckman et al. 2024).

Over the past decade, reports of *L. garvieae* infections have increased, likely due to improved diagnostic tools and greater clinical awareness (Malek et al. 2019; Lee et al. 2023).

Distinguishing *L. garvieae* from *L. petauri* and *L. formosensis* is challenging due to their high phenotypic and biochemical similarity, frequent misidentification in MALDI-TOF MS databases, and the close similarity of their 16S rRNA sequences (Chen et al. 2013, 2014; Luo et al. 2015; Goodman et al. 2017; Altinok et al. 2022; Stoppani et al. 2023; Heckman et al. 2024).

The first confirmed *L. petauri* outbreak occurred in 2007 in Greek farmed rainbow trout and was retrospectively reclassified through genomic analysis (Savvidis et al. 2007; Kotzamanidis et al. 2020). Until 2016, cases of piscine lactococcosis in the South and North American continents were rare and isolated (Nelson et al. 2016). In recent years, however, the disease has emerged as a serious threat, with outbreaks reported in Brazil, Mexico, Canada and several areas of the United States (Heckman et al. 2024). In 2020, severe outbreaks in Southern California led to the culling of over 3.2 million trout; the isolates, initially identified as *L. garvieae*, were later confirmed as *L. petauri* via whole-genome sequencing (Shahin et al. 2021; de Ruyter et al. 2023; Heckman et al. 2024). Retrospective studies confirmed that previous cases of human infection, food contamination, or fish disease attributed to *L. garvieae* have instead involved these recently described species (Chan et al. 2024; Esposito, Bignami, et al. 2025; Esposito, Colussi, et al. 2025).

Current knowledge of *L. garvieae* virulence factors is limited, and even less is known about *L. petauri* and *L. formosensis* (Abraham et al. 2023). Studies have reported differences in the virulence gene content of *L. garvieae* strains from human, fish and food sources, indicating potential host-specific pathogenicity (Abraham et al. 2023). However, no systematic comparison has yet been conducted on the virulence determinants of the three species.

This study reports the first detection, to the best of our knowledge based on reference analysis of major scientific databases and grey literature, of *L. formosensis* subsp. *formosensis* in farmed rainbow trout in Europe. The bacterium was isolated from a single specimen exhibiting mild clinical signs in Northern Italy, providing preliminary insights into its possible geographical distribution, host range and potential risk to European aquaculture.

2 | Materials and Methods

2.1 | Study Site

The study was conducted in a trout farm located in the southern area of the Piedmont region, in Northern Italy (45°05′47″ N, 07°29′52″E; Figure S1). The facility is located in a rural area with a high density of agricultural and livestock activities characterised by a semi-continental climate and high seasonal variation in water temperature.

The facility comprises 8 concrete raceways, each measuring approximately 55 m in length and 7.2 m in width, with an average depth of 1.2 m. Water quality parameters [temperature (°C), dissolved oxygen (mg/L) and pH] are monitored in situ using a multiparameter probe (HI98194, HANNA instruments).

Fish were reared at a density of about 20 kg/m^3 and fed a commercial pelleted diet. All production stages, from fingerlings to market size, were represented at the facility, with regular stocking and harvesting cycles.

2.2 | Sampling, Gross Pathology and Diagnostic Analyses

Between August and September 2024, a total of 70 rainbow trout were sampled for routine health monitoring within the framework of the project "RESILTROUT: aquaculture resilient to global changes: research to support the Italian trout supply chain" (MASAF Decree No. 399082 of 28 July 2023).

The health status of the collected fish was evaluated through external and internal examinations. Parasitological and bacteriological assessments were performed according to standard diagnostic procedures (Noga 2010).

For bacteriology, samples from anterior kidney, spleen, brain and vitreous humour were aseptically streaked on Columbia Agar (CA) (Liofilchem, Italy) supplemented with 5% sheep blood, and on Tryptic Soy Agar (TSA) prepared in-house. Plates were incubated at 22°C ± 2°C for 72 h with daily evaluation. Dominant colonies were subcultured on CA and identified biochemically using the API ID 32 Strep system (bioMérieux, Marcy l''Etoile, France) according to the manufacturer's instructions. Identification was further confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics Inc., Billerica, MA, USA), followed by molecular characterisation (16S-23S rRNA ITS region, and virulence factors) (see Sections 2.4 and 2.5). To phenotypically discriminate between *L. formosensis* subsp. *bovis* and *L. formosensis* subsp. *formosensis*, growth was assessed on TSA supplemented with 4% NaCl prepared in-house, and on

GranuCult prime MRS agar (de Man, Rogosa e Sharpe) at 42°C (Chan et al. 2024).

2.3 | Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MICs) of the dominant pure bacterial cultures (tentatively identified as *Lactococcus* spp.) against eight antimicrobials was determined using MIC Test Strips (Liofilchem, Italy), following the protocol proposed by Öztürk et al. (2024). After subculturing on TSA for 24 h, colonies were transferred to brain heart infusion (BHI) broth and suspended in saline to achieve a standardised inoculum equivalent to 0.5 McFarland turbidity. MIC testing was performed for the following antibiotics: amoxicillin, ampicillin, florfenicol, gentamicin, penicillin, streptomycin, trimethoprim/sulfamethoxazole and tetracycline (Bioanalyse, Ankara, Turkey). Antibiotic concentrations on the strips ranged from 0.016 to 256 μg/mL, except for penicillin and trimethoprim/sulfamethoxazole, which ranged from 0.002 to 32 μg/mL. Quality control (QC) reference strains were employed, in accordance with CLSI guidelines (CLSI 2015).

2.4 | Molecular Identification

Genomic DNA obtained from four isolates from the present study and two additional isolates previously isolated in natural piscine lactococcosis outbreaks (included for pathogenicity factors analysis and identified as 9756 and 5793) was extracted using a combined boiling and freeze–thaw protocol (Pastorino et al. 2021). The 16S-23S rRNA ITS region was amplified by end-point PCR and sequenced as described by Stoppani et al. (2023).

Sequence data were analyzed with DNASTAR Lasergene software and compared to GenBank entries via BLAST for identification.

2.5 | Molecular Serotyping and Virulence Gene Evaluation

Serotypes were identified by PCR according to the protocol described by Ohbayashi et al. (2017). Virulence-associated genes were screened through two multiplex and one simplex PCR assays following Salighehzadeh et al. (2020). Additional PCR assays targeting capsule gene cluster components and haemolysins (*hly-1*, *hly-2*, *hly-3*) followed protocols described by Ture and Altinok (2016) and Teker et al. (2019) respectively.

2.5.1 | External Dataset Used for Comparative Analysis of Virulence Factors

To contextualise our findings, we incorporated an external dataset described by Chan et al. (2024), which includes virulence factor results from 223 additional strains (including 11 listed in Table 3). These isolates, collected between 1974 and 2025, originated from various geographical regions (Africa, Asia, Europe and North America); although the origin of two samples could not be determined (Chan et al. 2024).

The dataset comprised different *Lactococcus* species: *Lactococcus garvieae* ($n = 43$), *Lactococcus petauri* ($n = 126$), *Lactococcus formosensis* subsp. *bovis* ($n = 42$) and *Lactococcus formosensis* subsp. *formosensis* ($n = 12$). For consistency with our study, only results corresponding to specific virulence genes were considered, i.e., the capsule gene cluster (*CGC*), adhesion genes (*adh*, *adhCI*, *adhCII*, *adhPavA*, *adhPsaA*, *LPxTG-2*, *LPxTG-3*), enzymes including *eno* (enolase), *pgm* (phosphoglucomutase) and *sod* (*superoxide dismutase*), as well as hemolysin genes (*hly-1*, *hly-2*, *hly-3*).

Isolates were classified into five source categories:

1. Freshwater fish [i.e., Rainbow trout (*Oncorhynchus mykiss*), Bighead carp (*Hypophthalmichthys nobilis*), Tilapia (*Oreochromis* spp.) and So-iuy mullet (*Planiliza haematocheilus*)];
2. Saltwater fish [i.e., Greater amberjack (*Seriola dumerili*), Cobia (*Rachycentron canadum*), European seabass (*Dicentrarchus labrax*), Gilthead seabream (*Sparus aurata*), Yellowtail amberjack (*Seriola lalandi*) and Large yellow croaker (*Larimichthys crocea*)];
3. Humans (faecal and clinical isolates);
4. Terrestrial mammals (i.e., bovines, domestic dogs);
5. Others (including food products, insects and miscellaneous sources).

In the dataset of Chan et al. (2024), *Lactococcus* isolates were predominantly from humans and freshwater fish, with *L. garvieae* mainly from humans and rainbow trout, *L. petauri* mostly from human faeces and rainbow trout and *L. formosensis* subsp. *bovis* almost exclusively from bovines (primarily mastitis cases), while subsp. *formosensis* included human, fish and environmental sources.

2.6 | Phylogenetic Analysis

The average pairwise Jukes-Cantor (JC) distance was calculated, and phylogeny was inferred using the Neighbour-Joining (NJ) method (Saitou and Nei 1987). A bootstrap test was performed with 1000 replicates to assess branch robustness (Felsenstein 1985). Evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al. 2004). All ambiguous positions were removed for each sequence pair using the pairwise deletion option. The cut-off value for the condensed tree was set at 50%, and branches with values $< 50\%$ were collapsed. All evolutionary analyses were performed using MEGA X (v. 10.2.6) (Kumar et al. 2018).

2.7 | Statistical Analysis

Data analysis was performed using R software (v. 4.4.2; R Core Team, 2024). Descriptive statistics were carried out, and visualisations were produced using the "ggplot2" package with the *ggplot()* function.

Multiple Correspondence Analysis (MCA) was performed using the "FactoMineR" and "factoextra" packages to conduct the

analysis and generate visualisations, respectively. MCA was preferred over Principal Component Analysis (PCA) because it is specifically suited for categorical data, such as the presence/absence (1/0) of virulence genes, providing a more appropriate and interpretable analysis of associations within the dataset.

2.8 | Ethics Statement

All fish were sampled *post-mortem* as part of a research project coordinated by the *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta* (IZSPLV). Specimens were received in good condition, refrigerated during transport and submitted to the Fish Disease and Genetics and Genomics Units at IZSPLV for diagnostic investigations.

All animal handling procedures complied European and Italian welfare regulations (Directive 2010/63/EU; Legislative Decree No. 26/2014).

The study was approved by the IZSPLV Ethics Committee under protocol no. IZSTO\I/U\0005147\16-05-2024, within the activities of the Institutional Framework and General Affairs Unit.

3 | Results

3.1 | Morphometric, Necropsy and Environmental Findings

While most of the examined fish appeared clinically healthy, four out of the 70 examined fish (5.7%) exhibited mild clinical and pathological signs of piscine lactococcosis. The affected specimens had a mean weight of 52.6 ± 22.5 g and a total length of 15.7 ± 2.1 cm. Specifically, these fish showed mild enteritis and mild splenomegaly (Figure 1a), with Figure 1b highlighting enteritis, ascitic fluid containing blood and mild splenomegaly. No other significant lesions were observed in other organs. No ecto- or endoparasites were detected during parasitological examination.

Water parameters were monitored during the sampling period and remained within optimal ranges for trout farming: pH of 7.0 ± 0.2, temperature of 17°C ± 1.5°C and dissolved oxygen of 8 ± 1 mg/L.

3.2 | Bacteriological, Biochemical and Molecular Analyses

Out of the 70 fish, four (IDs: 42678.5.1; 42678.5.2; 99,739/6; 99,751/5) were positive for *Lactococcus garvieae* by bacteriological examination. MALDI-TOF MS identified them as *L. garvieae*/*L. petauri*/*L. formosensis* (score 2.19–2.21), without discriminating among the three species.

Biochemical profiling confirmed previously reported patterns (Vela et al. 2024): all *L. petauri* isolates hydrolysed hippurate and produced acid from sucrose and tagatose; *L. garvieae* isolates did not hydrolyse hippurate or acidify sucrose, with variable acid production from tagatose. The *L. formosensis* isolate showed a distinct biochemical profile (Table 1), and one isolate was not matched in the APIweb database, though the closest profile suggested *L. garvieae*. API Rapid ID 32 STREP V4.0 assigned a numerical profile of 30,223,711,031.

Sequencing of the 16S-23S rRNA ITS region identified isolate 99,751/5 as *L. formosensis* subsp. *formosensis* (100% identity with reference PP872542; GenBank PV857758). Other isolates were identified as *L. garvieae* (99,739/6, 99.81%; 42678.5.1, 99.13%; GenBank AP009332) and *L. petauri* (42678.5.2, 100%; GenBank CP141697).

3.3 | Phylogeny

JC distance was 0.02, supporting NJ tree application. The resulting tree (Figure 2) included 28 sequences ($n = 15$ *Lactococcus petauri*, $n = 3$ *L. formosensis*, $n = 9$ *L. garvieae*, $n = 1$ *L. lactis* outgroup). Three main clades were observed: *L. petauri* (with a separate human strain), *L. formosensis* subsp. *formosensis* and *L. garvieae*, with the outgroup *L. lactis*. Isolate 99,751/5 clustered within the *L. formosensis* clade, confirming identity from BLAST on 16S-23S rRNA ITS region analysis.

3.4 | Antimicrobial Susceptibility

The MIC of isolate 99,751/5 showed susceptibility to all tested antibiotics. No MIC reference values exist for this subspecies; therefore, results were compared with published MIC50, MIC90

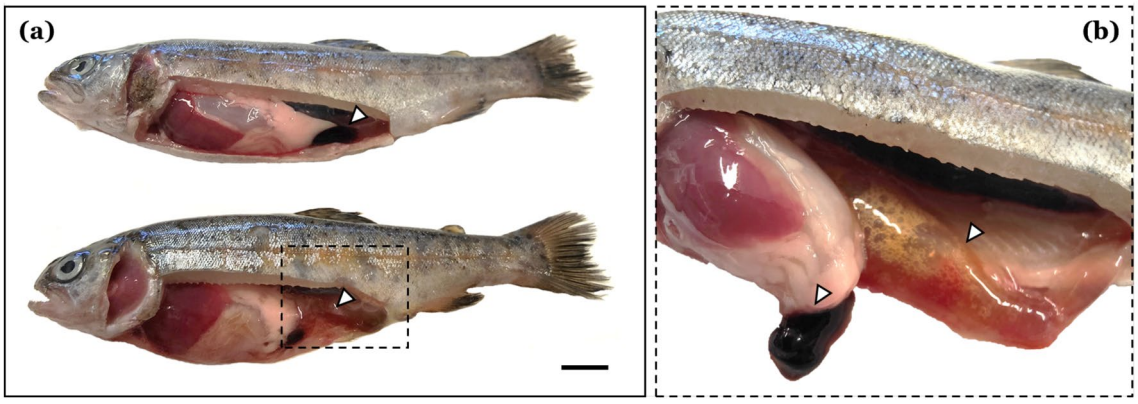

FIGURE 1 | Specimens positive for *Lactococcus* spp. exhibiting mild clinical signs (a). White arrows indicate mild splenomegaly and mild enteritis (a). Detail of enteritis, ascitic fluid which contain blood and splenomegaly (b). Scale bar: 1 cm.

TABLE 1 | Biochemical profile (API Rapid ID 32 Strep) of *Lactococcus formosensis* subsp. *formosensis* isolated from the kidney of rainbow trout (present study), with comparison to *Lactococcus garvieae* and *Lactococcus petauri* isolates from fish.

Reference	99739/6[a]	Bondavalli et al. (2024)	Present study
Species	*Lactococcus garvieae*	*Lactococcus petauri*	*Lactococcus formosensis* subsp. *formosensis*
Host	Rainbow trout (*Oncorhynchus mykiss*)	Pumpkinseed (*Lepomis gibbosus*)	Rainbow trout (*Oncorhynchus mykiss*)
Reactions/Enzymes			
Arginine DiHydrolase (ADH)	+	+	+
β GLUcosidase (βGLU)	+	+	+
β GALactosidase (βGAR)	−	−	−
β GlUcURonidase (βGUR)	−	−	−
α glycoconjugates (αGAL)	−	+	−
4-nitrophenyl-ßD-galactopyranoside2-CHA (PAL)	−	−	−
D-ribose (RIB)	−	+	−
D-mannitol (MAN)	+	+	+
D-sorbitol (SOR)	−	−	−
D-lactose (bovine origin) (LAC)	−	+	−
D-trehalose (TRE)	+	+	+
D-raffinose (RAF)	−	+	−
Sodium pyruvate (VP)	+	+	+
L-alanyl-L-phenylalanyl-L-prolineß-naphthylamide (APPA)	+	+	+
2-naphthyl-ßD-galactopyranoside (βGAL)	−	+	−
Pyroglutamic acid-ß-naphthylamide (PyrA)	+	+	+
6-bromo-2-naphthyl-N-acetylßD-glucosaminide (βNAG)	+	+	+
L-glycyl-L-tryptophanß-naphthylamide (GTA)	+	+	+
Sodium hippurate (HIP)	−	+	+
Glycogen (GLYG)	−	−	−
Pullulan (PUL)	−	−	−
D-maltose (MAL)	+	+	+
D-melibiose (MEL)	−	−	−
D-melezitose (MLZ)	−	−	−
D-saccharose (SAC)	−	+	−
L-arabinose (LARA)	−	−	−
D-arabitol (DARL)	−	−	−
Methyl-ßD-glucopyranoside (MβDG)	−	+	+
D-tagatos (TAG)	−	+	+

(Continues)

TABLE 1 | (Continued)

Reference	99739/6[a]	Bondavalli et al. (2024)	Present study
4-nitrophenyl-βD-mannopyranoside (βMAN)	−	−	−
Cyclodextrin (CDEX)	+	+	+
Urea (URE)	−	−	−

Note: +, Positive; −, negative.
[a]Field strain from IZSPLV archive.

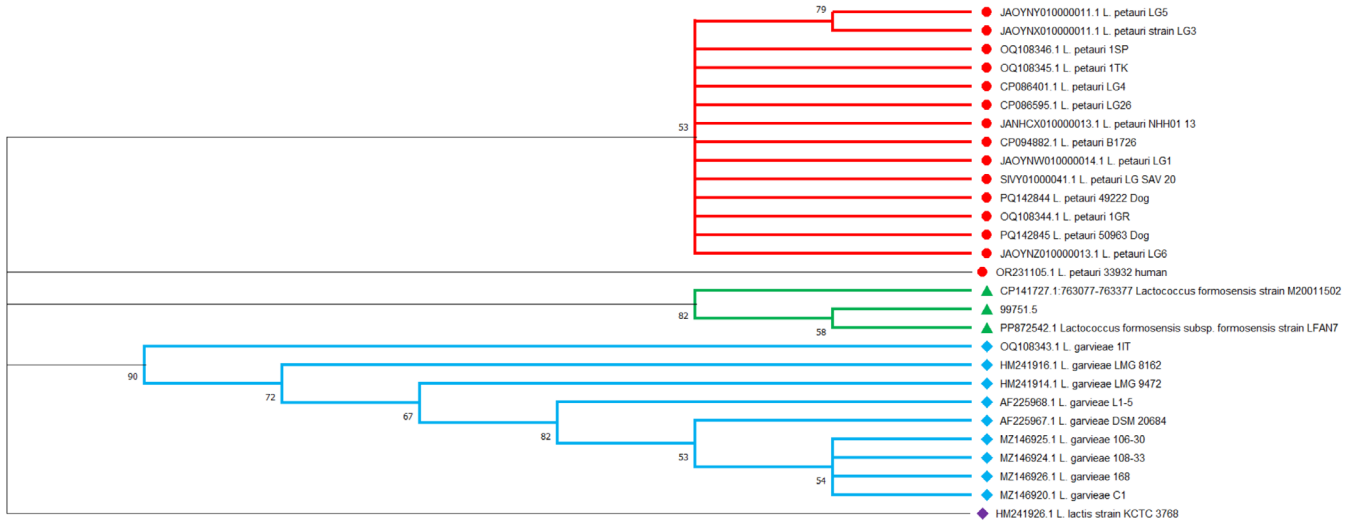

FIGURE 2 | The optimal phylogenetic tree with evolutionary distances is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The red clade indicates *Lactococcus petauri* (circle), the green clade *Lactococcus formosensis* subsp. *formosensis* (triangle), while blue clade is *Lactococcus garvieae* (diamond). Purple represents the outgroup (diamond).

TABLE 2 | Comparison of MIC values obtained in this study with MIC50, MIC90 and ECV99 reported by Öztürk et al. (2024); Lp = *Lactococcus petauri*; Lg = *Lactococcus garvieae*; Lf-f = *Lactococcus formosensis* subsp. *formosensis*; N/A = not available; WT = wild-type.

Antibiotic	MIC strip (Lf-f; this study)	MIC50/MIC90 (μg/mL)	ECV99 (μg/mL)	Interpretation
Amoxicillin (AMX)	0.19	0.5/1 (Lp) 0.25/1 (Lg)	1.25 (Lp)/2.51 (Lg)	WT
Ampicillin (AMP)	0.38	0.5/2 (Lp) 1/2 (Lg)	5.01 (Lp)/2.51 (Lg)	WT
Florfenicol (FFC)	4	7/64 (Lp) 1/8 (Lg)	40.12 (Lp)/20.06 (Lg)	WT
Gentamicin (CN)	0.25	1/4 (Lp) 2/32 (Lg)	10.03 (Lp)/5.01 (Lg)	WT
Penicillin (P)	0.38	0.5/1 (Lp) 1/32 (Lg)	10.03 (Lp)/2.51 (Lg)	WT
Streptomycin (S)	8	4/16 (Lp) 1/32 (Lg)	160.49 (Lp)/80.24 (Lg)	WT
Trimethoprim/Sulfamethoxazole (SXT)	0	32/32 (both)	N/A	WT
Tetracycline (TE)	1	4/8 (Lp) 1/2 (Lg)	5.01 (both)	WT

TABLE 3 | Comparison of virulence genes between *Lactococcus petauri*, *Lactococcus garvieae* and *Lactococcus formosensis* subsp. *formosensis*.

Bacteria	***Lactococcus petauri***					***Lactococcus garvieae***				***Lactococcus formosensis* subsp. *formosensis***
Species	Rainbow trout	Rainbow trout	European seabass	Domestic dog	Human	Rainbow trout	Rainbow trout	Rainbow trout	Gilthead seabream	Rainbow trout
Location	Northeast Italy	Northwest Italy	Central Italy	Northwest Italy	Northwest Italy	Northwest Italy	Northwest Italy	Northeast Italy	Central Italy	Northwest Italy
Reference	Unpublished data	Present study	Esposito, Colussi, et al. 2025	Sciuto et al. 2024	Colussi et al. 2023	Present study	Present study	Present study	Esposito, Bignami, et al. 2025	Present study
ID	9756	42678/5.2	13332/2.1	49222–50963	33932	99739/6	42678/5.1	5793	80214/10.22	99751/5
Serotype	I	I	I	I	I	I	I	I	I	II
CGC	−	−	−	−	−	−	−	−	−	−
Adhesion genes										
adh	−	−	−	−	+	−	+	+	+	−
adhCI	+	+	+	+	+	+	+	+	+	+
adhCII	−	−	−	−	−	−	−	−	+	−
adhPavA	+	+	+	+	+	+	+	−	+	−
adhPsaA	+	+	+	+	+	+	+	−	+	+
LPxTG-2	+	+	−	−/+	−	−	+	−	−	+
LPxTG-3	+	+	+	+	−	+	+	−	+	−
Enzyme genes										
eno	+	+	+	−	+	+	+	+	+	+
NADH oxidase	−	−	−	−	+	−	−	+	+	−
pgm	−	−	+	−	+	−	−	−	+	−
sod	+	+	+	+	+	+	+	+	+	+

(Continues)

TABLE 3 | (Continued)

Bacteria	*Lactococcus petauri*				*Lactococcus garvieae*				*Lactococcus formosensis* subsp. *formosensis*
Hemolysin genes									
hly-1	−	−	−	−	+	+	+	+	−
hly-2	+	+	+	+	+	+	+	+	+
hly-3	−	−	−	−/+	+	+	+	+	−

Abbreviations: *adh*, adhesin; *adhCI*, adhesin cluster 1; *adhCII*, adhesin cluster 2; *CGC*, capsule gene cluster; *eno*, enolase; *hly-1*, hemolysin 1; *hly-2*, hemolysin 2; *hly-3*, hemolysin 3; *pgm*, phosphoglucomutase; *sod*, superoxide dismutase.

and ECV99 values for *Lactococcus petauri* and *L. garvieae* (Öztürk et al. 2024) as a preliminary interpretation (Table 2). MICs for β-lactams ranged from 0.19 to 0.38 μg/mL, florfenicol 4 μg/mL, gentamicin 0.25 μg/mL, tetracycline 1 μg/mL and streptomycin 8 μg/mL, all below wild-type thresholds.

3.5 | Serotyping and Virulotyping

The *L. formosensis* isolate belonged to serotype II. Capsule gene cluster (CGC) was absent. Adherence genes *adhCI*, *adhPsaA* and *LPxTG-2* were present; *adh*, *adhCII*, *adhPavA* and *LPxTG-3* were absent. Enzymatic factors *eno* and *sod* were identified; *pgm* and *NADH oxidase* were not detected. Only *hly-2* was present among haemolysins (Table 3).

3.5.1 | Comparative Analysis of Virulence Factors

Comparison with global datasets (Chan et al. 2024) revealed species- and host-specific virulence patterns (Figures 3, 4, S2, S3). *Lactococcus formosensis* subsp. *bovis* consistently lacked *adh* and *LPxTG-3* (*LPxTG-2* detected in one isolate), while subsp. *formosensis* from freshwater fish lacked *adh*, *adhCII* and *LPxTG-3*, showing variable *LPxTG* motifs; saltwater fish isolates showed a similar pattern but carried all other virulence genes. *Lactococcus garvieae* maintained high prevalence of most virulence genes across hosts, with minor absences for *adh* and *LPxTG* proteins. *Lactococcus petauri* showed widespread absence of *adh*, partial absence of *LPxTG* proteins and sporadic gaps in *adhCI*, *adhCII* and *eno*, while most other genes were consistently present.

MCA revealed clustering by species, host and geography: aquatic isolates were distinct from terrestrial and human isolates, with *L. garvieae* associated with European freshwater, *L. formosensis* with Asian origins and terrestrial mammals and *L. petauri* showing broader host and ecological distribution, highlighting clear ecological and taxonomic patterns despite modest variance (Dim1: 6.9%, Dim2: 4.5%).

4 | Discussion

To the best of our knowledge, this study reports the first detection of *Lactococcus formosensis* subsp. *formosensis* in rainbow trout in Europe. Current reports indicate that *L. formosensis* has been mainly reported in Asia and South America (Barbanti et al. 2024; Chan et al. 2024; Heckman et al. 2024).

Only one of the 70 sampled fish was positive for *L. formosensis* subsp. *formosensis* and exhibited mild clinical signs, while three other fish with mild signs were positive for *L. garvieae* or *L. petauri*. This observation is consistent with evidence that young trout (< 80 g) may act as subclinical carriers without triggering overt disease, even though experimental infections can reproduce piscine lactococcosis (Vendrell et al. 2006). No outbreak occurred during the observation period, and the study was conducted under routine farm monitoring conditions.

Comparative virulotyping revealed species- and host-specific patterns. *L. formosensis* subsp. *formosensis* showed variable

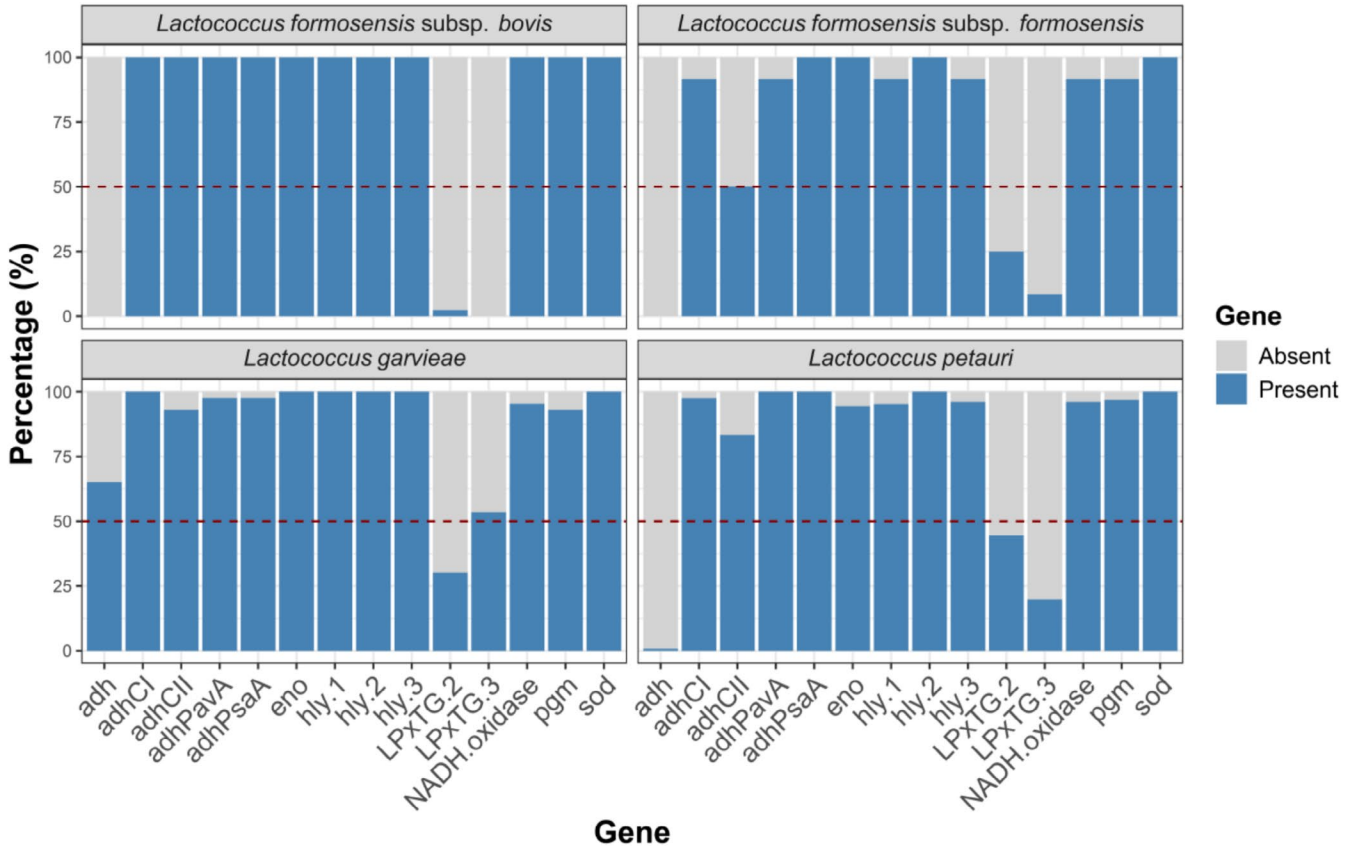

FIGURE 3 | Comparative overview of virulence gene presence across *Lactococcus* species. For each gene, blue bars indicate the percentage of isolates in which the gene is present. A red dashed line marks the 50% prevalence threshold.

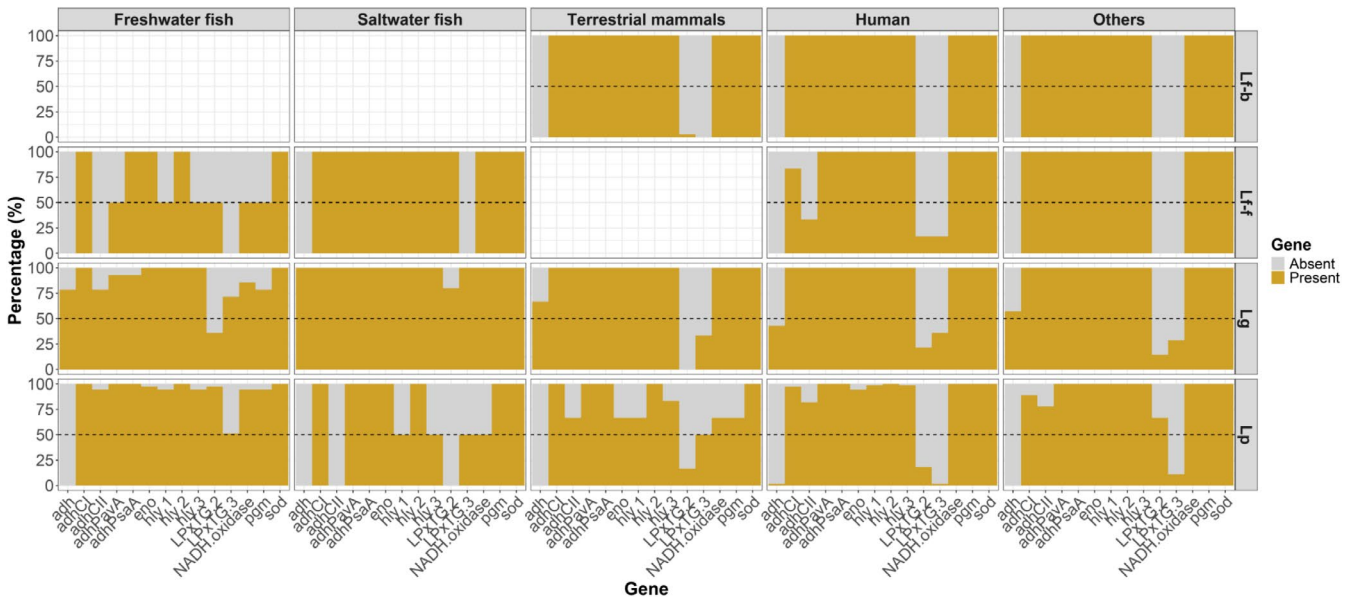

FIGURE 4 | Percentage distribution of virulence genes across bacterial isolates by species and host category. Yellow bars: Positive isolates; grey bars: Negative isolates. Hosts: Freshwater fish, saltwater fish, terrestrial mammals, humans, others. Black dashed line: 50% threshold. Species abbreviations: Lg = *L. garvieae*, Lp = *L. petauri*, Lf-b = *L. formosensis* subsp. *bovis*, Lf-f = *L. formosensis* subsp. *formosensis*.

presence of adhesion and *LPxTG* genes, while *L. garvieae* maintained high prevalence of most virulence genes, and *L. petauri* showed sporadic gaps in adhesion genes (Chan et al. 2024). MCA analysis highlighted clustering by species, host and geography, with aquatic isolates distinct from terrestrial and human isolates. *L. garvieae* clustered with European freshwater, *L. formosensis* with Asian origins and terrestrial mammals and *L. petauri* displayed broader host and ecological

distribution. These patterns are consistent with previously reported data on *sod*, *eno*, *adhCI*, *AdhPsaA*, *AdhPav*, *hly-2* and *CGC* (Chan et al. 2024).

Serotyping confirmed serotype II for the *L. formosensis* isolate, consistent with its species-specific association (Mahmoud et al. 2023; Barbanti et al. 2024), while all other isolates were serotype I.

The antimicrobial susceptibility profile of the *L. formosensis* isolate was consistent with wild-type ranges reported for *L. petauri* and *L. garvieae* (Öztürk et al. 2024). MICs for β-lactams, florfenicol, gentamicin, tetracycline and streptomycin were below ECV99 thresholds, indicating the absence of acquired resistance traits. These results align with previous observations that isolates from low-antimicrobial-pressure environments show greater susceptibility (Ture and Boran 2015; Duman et al. 2020).

Overall, the findings highlight that young rainbow trout can carry *L. formosensis* subsp. *formosensis* without severe clinical signs and emphasise the importance of targeted surveillance and advanced diagnostics to clarify its epidemiology and potential risk to European aquaculture.

5 | Conclusion

This study reports the first detection and isolation of *Lactococcus formosensis* subsp. *formosensis* in rainbow trout in Europe. The isolate was recovered from a single fish showing mild clinical signs, and its virulence profile showed fewer known factors compared with *L. petauri*, despite considerable genetic similarity. These findings provide preliminary information on the presence of *L. formosensis* subsp. *formosensis* in European aquaculture and highlight the importance of continued monitoring. Future research should focus on its pathogenic potential, ecological role and interactions with other microbial communities to support surveillance and risk assessment. In vivo infection trials are needed to clarify pathogenicity and host interactions.

Author Contributions

Silvia Colussi: conceptualization (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); supervision (equal); writing – original draft preparation (equal); writing – review and editing (equal). **Giuseppe Esposito:** conceptualization (equal); data curation (lead); formal analysis (lead); investigation (equal); methodology (equal); supervision (equal); writing – original draft preparation (equal); writing – review and editing (equal). **Khalid Shahin:** conceptualization (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Pier Luigi Acutis:** investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Lucio Fariano:** conceptualization (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Claudio Ghittino:** conceptualization (supporting); investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Maria Goria:** conceptualization (supporting); investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Fabio Bondavalli:** investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Paolo Ajmone Marsan:** conceptualization (supporting); investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Giorgia Bignami:** investigation (supporting); methodology (supporting). **Elena Bozzetta:** conceptualization (supporting); investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Marino Prearo:** conceptualization (supporting); investigation (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Paolo Pastorino:** conceptualization (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); supervision (equal); writing – original draft preparation (equal); writing – review and editing (equal).

Acknowledgements

The authors wish to thank Prof. Esteban Soto Martinez (University of California, Davis, UCD) for his valuable suggestions and insightful advice, which greatly contributed to the improvement of the manuscript. We also thank the fish farmers for their availability and assistance during all phases of sampling. Open access funding provided by BIBLIOSAN.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** a) Geographical map of Italy (highlighted in grey); b) the study area highlighted in green (Piedmont region, north-western Italy. **Figure S2:** Heatmap showing the comparative prevalence of virulence genes in *Lactococcus* species. Rows and columns are hierarchically clustered to highlight patterns of gene presence. Numeric values inside the cells represent the exact percentage of prevalence. **Figure S3:**. Multiple correspondence analysis (MCA). *Lactococcus garvieae* = Lg, *Lactococcus petauri* = Lp, *Lactococcus formosensis* subsp. *bovis* = Lf-b, *Lactococcus formosensis* subsp. *formosensis* = Lf-f.