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Case Report

First Report of *Lactococcus petauri* in the Pumpkinseed (*Lepomis gibbosus*) from Candia Lake (Northwestern Italy)

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Abstract: *Lactococcus petauri* has emerged as a pathogen affecting farmed fish. In this case report, *L. petauri* was isolated from *Lepomis gibbosus* captured in Lake Candia, located in Piedmont, Northwestern Italy. Initially identified as *L. garvieae* using conventional methods like RAPID 32 ID STREP and MALDI-TOF, molecular analysis based on sequencing of the 16S-23S rRNA internal transcribed spacer region (ITS) confirmed it as *L. petauri*. The study also assessed the isolated strain's pathogenicity by examining hemolysin and capsule cluster genes, revealing only hemolysin 2 amplicon; notably, the fish exhibited no lesions or pathological alterations. The *L. petauri* strain from this study displayed resistance to several antibiotics, being sensitive only to ampicillin, amoxicillin, florfenicol, and G-penicillin. This research provides new insights into host range of *L. petauri* and raises awareness of potential public health implications, particularly concerning zoonotic potential.

Keywords: antibiotic resistance; ITS; hemolysin 2; *Streptococcaceae*; *Lactococcus petauri*; *Lepomis gibbosus*

Key Contribution: *L. petauri* is reported for the first time in fish species in Italy; in particular, it is reported in wild pumpkinseed capture in Lake Candia located in Piedmont.

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1. Introduction

Lactococcosis is a disease encountered in a wide variety of fish species causing mortalities and having great economic impact on farmed fish [1]. *Lactococcus garvieae* has been considered for a long time the only causative agent of the disease.

Lactococcus petauri sp. nov. 159469, isolated from facial abscess of sugar glider (*Petaurus breviceps*), was first described as a new species of the *Lactococcus* genus by Goodman et al. [2]. Phylogenetic and genotyping analysis revealed that this novel distinct species shares most of its genome with *L. garvieae*.

In 2019, it was isolated in rainbow trout (*Oncorhynchus mykiss*) affected by lactococcosis in Greece [3]; it was later described in the United States [4,5], Turkey [6], Spain [7,8], and in Nile tilapia (*Oreochromis niloticus*) in Brazil [9], but it was never observed in reared and wild fishes in Italy. Nevertheless, recently a case of human urinary tract infection caused by *L. petauri* was reported in Italy [10].

Lactococcosis is a serious systemic disease, with a hyperacute–acute course, which causes serious losses of the affected fish stocks [11]; it is particularly pathogenic for rainbow trout, causing high mortalities in a short time [1,12–14]. The clinical signs of diseased fish are lethargy, erratic swimming, evident bilateral exophthalmos, and cutaneous melanosis [1,11]; affected fish show very early anorexia [15]; the anatomopathological analysis highlights

the presence of diffuse hemorrhages in the liver, perivisceral fat, swim bladder, evident splenomegaly, and catarrhal or hemorrhagic-catarrhal enteritis [1,11].

Lactococcus petauri is very similar to *L. garvieae* considering phenotypical and genetical characteristics, and the two species cannot be distinguished by methods commonly used as routine tests, such as commercial identification systems, MALDI-TOF, or PCR assays targeting the 16S rRNA gene [7]. Genome comparison demonstrated, in fact, that the identification of several isolates of *L. garvieae* previously to the description of *L. petauri*, was incorrect [3,6]. Since genomic analysis is expensive and laborious, a rapid and less expensive diagnostic system has been developed based on the sequencing of the 16S-23S intergenic rRNA spacer region (ITS 16S-23S) [7] and on multiplex PCR based on the *TagG* gene, which is a part of the ABC transporter complex TagGH [16].

This study aims to report the first finding of *L. petauri* isolated in a wild fish (*Lepomis gibbosus*) caught in a morainic natural lake (Lake Candia) located in Northwestern Italy.

2. Materials and Methods

2.1. Study Area

Lake Candia, situated in Piedmont (Northwestern Italy; coordinates 45°19′28″ N, 7°54′35″ E), holds significant importance as a wetland. Positioned close to the Western Alps, the Lake Candia Nature Park is officially recognized as a designated biotope in Piedmont and holds the classification of a Site of Community Importance (SCI-IT1110036) under the European Union's Habitats Directive (92/43/EEC). Over 200 bird species, along with amphibians such as *Bufo bufo* and *Rana dalmatina*, reptiles, and fish have been documented in this lake [17].

2.2. Fish Sampling

The fish were caught as part of a monitoring campaign for *Procambarus clarkii* in Lake Candia. In accordance with national rules, invasive alien species must be contained and removed from the environment; *L. gibbosus* falls into this category.

Fish were captured using a cylindrical trap designed for crayfish (with dimensions of 90 cm in length, 1 cm mesh size, 30 cm cross-section, and two funnels each 10 cm in diameter) baited with canned cat food. These traps were positioned at mid-depth levels (ranging from 0.5 to 2 m) across Lake Candia and retrieved after a 24 h period. Following the retrieval, fish were taken out and transported to the Fish Diseases Laboratory of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta in compliance with existing regulations. All sampled fish (No = 20) belonged to the species *Lepomis gibbosus*.

2.3. Sanitary Monitoring

Fish were necropsied and processed for parasitological and bacteriological analyses. For the parasitological analysis, samples of tissue scrapings taken from both skin and gill filaments were processed using a water drop, covered with a fresh cover slip to create a wet mount preparation. Subsequently, the prepared slides were scrutinized under an optical microscope (Olympus BX40, Olympus, Hamburg, Germany) at magnifications ranging from 10× to 40×. Bacteriological analysis was performed on the kidney, eye, and brain. The inoculum was directly plated out on first isolation media (Columbia Blood Agar) on the same day of recovery from the crayfish trap. The samples were incubated at 22 ± 2 °C for 24–72 h; the colonies were cloned and identified biochemically by using the Rapid ID32 Strep system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions and by using mass spectrometry MALDI-TOF (Bruker, Billerica, MA, USA). Furthermore, DNA extraction was carried out using the boiling freeze–thaw method. The bacterial colony was diluted in 200 µL of DNA-free water and the sample was boiled at 95 °C. After 10 min, the sample was frozen and then thawed at 4 °C. The sample was centrifugated at 10,000 rpm for 1 min and 100 µL of supernatant was collected in a sterile Eppendorf. The biochemical characterization of the isolate was evaluated using API RAPID ID 32 STREP (bioMérieux, Marcy l'Etoile, France).

2.4. Antibiotic Resistant Profile of the Isolate

Antibiotic resistance of the bacterial strain under investigation was also tested using the Kirby–Bauer method [18]. The following antimicrobials were tested: amoxicillin (25 µg), ampicillin (2 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), gentamicin (2 µg), kanamycin (30 µg), oxytetracycline (30 µg), G-penicillin (10 µg), spiramycin (100 µg), streptomycin (10 µg), tetracycline (30 µg), and thiamphenicol (30 µg) (Bioanalyse, Ankara, Turkey). The isolate was introduced into Mueller–Hinton agar plates by streaking the entire surface in three different directions, and rotating the plate at 60° angles after each streak; antimicrobial discs were dispensed onto the plates using sterile forceps. Following overnight incubation at 22 °C, the resulting zones were assessed based on the minimum inhibition diameter. The diameter of inhibition of each antimicrobial agent was then classified as susceptible, intermediate, or resistant, in accordance with the interpretative criteria outlined by the Clinical and Laboratory Standards Institute (CLSI) and the breakpoint reported in the study on *L. petauri* conducted by Cataõ Egger and colleagues in 2022 [9] (Table S1, Supplementary Materials). The evaluation criteria from VET03-A [19] and VET03/VET04-S2 [20] were employed for testing bacteria isolated from aquatic animals.

2.5. Molecular Analyses

Genetic analyses were aimed at discerning *L. garvieae* from *L. petauri*. The 16S-23S rRNA internal transcribed spacer (ITS) region was amplified by PCR using the protocol described by Stoppani et al. [7]. The 16S-23S rRNA ITS region was amplified by PCR using the primers 16S 5′-GCTGGATCACCTCCTTTCT-3′ and 23S 5′-GGTACTTAGATGTTTCAGTTCC-3′ described by Kabadjova et al., 2002 [21]. Amplicons were purified with a Qiaquick purification kit (Qiagen, Hileden Germany) and sequenced using the chemistry of Brilliant Dye Terminator (v1.1) (NimaGen, Nijmegen, Netherlands) on the genetic analyzer (Applied Biosystems 3130XL, Thermo Fisher, Carlsbad, USA). Alignment was carried out using DNASTAR Lasergene Software. Consensus sequence was compared with nucleotide sequences in the GenBank database using the basic local alignment search tool (BLAST). Moreover, hemolysins and capsule cluster genes were evaluated as pathogenicity factors. The three hemolysin genes (*Hly1*, *Hly2*, and *Hly3*) were amplified using the protocol previously described by Teker et al. [22]. Amplification of capsule cluster genes were carried out as reported by Ture and Altinok [23] and revealed on 2% agarose gel.

3. Results

3.1. Phenotypic Characteristics of the Isolate

Necropsy and parasitological inspection yielded negative results in all specimens and no pathological signs were found. Bacteriological analysis was positive only from the eye of one specimen. Analysis by MALDI-TOF allowed to identify the isolate as *L. garvieae* (identification score: 2.19). Biochemical identification of the isolate using the APIweb database (bioMérieux, France) yielded *Enterococcus gallinarum* (99.5%). Table 1 summarizes the biochemical features of the isolate (RAPID ID 32 STREP). Generally, the isolate uses L-arginine, resorufin-ßD-glucopyranoside, L-alanyl-L-phenylalanyl-L-proline-ß-naphthylamide, 2-naphthyl-ßD-galactopyranoside, pyroglutamic acid-ß-naphthylamide, 6-bromo-2-naphthyl-N-acetyl-ßD-glucosaminide, and L-glycyl-L-tryptophan-ß naphthylamide; it produces acid from ribose, glucose, mannitol, lactose, raffinose, trehalose, saccharose, cyclodextrin, methyl-ßD-glucopyranoside, D-maltose, and D-tagatose. No acid was produced from resorufin-ßD-galactopyranoside, 4-nitrophenyl-D-galactopyranoside, pullulan, D-melibiose, D-sorbitol, L-arabinose, D-arabitol, D-melezitose, or glycogen. The isolate did not use urea as substrate but produced acetoin (VP test) and hydrolyzed the sodium hippurate.

The results of the Kirby–Bauer test for antibiotic resistance are summarized in Table 2. The isolate was sensitive to ampicillin, amoxicillin, florfenicol, and G-penicillin. The isolate yielded intermediate sensitivity to enrofloxacin, while it showed resistance to

the other molecules (erythromycin, gentamycin, kanamycin, streptomycin, spiramycin, oxytetracycline, tetracycline, and thiamphenicol).

Table 1. Biochemical features (API RAPID ID 32 STREP) of *Lactococcus petauri* isolated from the eye of *Lepomis gibbosus*. "+" = positive; "−" = negative.

Test	Result
Arginine DiHydrolase (ADH)	+
ß GLUcosidase (βGLU)	+
ß GALactosidase (βGAR)	−
ß 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)	+
D-saccharose (SAC)	+
L-arabinose (LARA)	−
D-arabitol (DARL)	−
Cyclodextrin (CDEX)	+
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)	−
Methyl-ßD-glucopyranoside (MβDG)	+
D-tagatos (TAG)	+
4-nitrophenyl-ßD-mannopyranoside (βMAN)	−
Urea (URE)	−

Table 2. Pattern (resistant, sensitive, intermediate) of antibiotic resistance profile of *Lactococcus petauri* isolated from the eye of *Lepomis gibbosus*.

Antibiotic	Dosage	Pattern	Inhibition Zone
Ampicillin	2 µg	Susceptible	21 mm
Amoxicillin	30 µg	Susceptible	19 mm
Erythromycin	15 µg	Resistant	14 mm
Enrofloxacin	5 µg	Intermediate	18 mm
Florfenicol	30 µg	Susceptible	21 mm
Gentamycin	2 µg	Resistant	10 mm
Kanamycin	30 µg	Resistant	7 mm
G-penicillin	10 µg	Susceptible	24 mm
Streptomycin	10 µg	Resistant	10 mm
Spiramycin	100 µg	Resistant	13 mm
Oxytetracycline	30 µg	Resistant	12 mm
Tetracycline	30 µg	Resistant	12 mm
Thiamphenicol	30 µg	Resistant	10 mm

3.2. Molecular Analysis

BLAST analysis revealed an identity percentage of 100% with *L. petauri* ITS sequences isolated from rainbow trout in Spain, Turkey, and Greece, deposited in GenBank database and identified by the following accession numbers: OQ108344, OQ108345, and OQ108346 [7]. Amplification of capsule cluster genes was negative. Amplification of hemolysin genes was negative for hemolysins 1 and 3 and positive for hemolysin 2 (Figure S1, Supplementary Material).

4. Discussion

Lactococcosis poses a significant challenge throughout the Mediterranean basin, resulting in substantial economic losses and decreased production, with mortality rates reaching up to 80% [11]. In various European countries, such as Greece [3], Turkey [6], and Spain [8], outbreaks of lactococcosis associated with *L. petauri* have been extensively documented. However, studies conducted by the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (Turin, Italy) indicate that only *L. garvieae* is prevalent in trout farms across Italy. Thus, a surveillance conducted on 62 Italian archival strains spanning from 1991 to 2021 only indicated the presence of *L. garvieae* at the national level [24]. Consequently, this study represents the first documentation of *L. petauri* in wild fish in Italy. This report, which involves a wild animal, is an important step towards a better understanding of the spread of this recently identified bacterium in Europe.

The genus *Lactococcus* in wild fish is poorly documented. A recent paper from 2022 reported a prevalence ranging from 3.85% to 9.76% in *Micropterus salmoides* and around 8% in *Lepomis macrochirus* from Californian lakes. Mortality or clinical signs were not reported in the analyzed samples [25]. Similar to that reported by Abraham et al. for *Lepomis macrochirus*, a prevalence of 5% in absence of mortality or clinical signs attributable to bacterium was reported in the present paper. As already stated in various papers, the possible transmission of pathogens through migratory birds, piscivorous birds, or other vectors has been reported several times [4,26] and may be one of the causes of introduction in uninfected areas.

Numerous clinical microbiology laboratories still use phenotypic techniques, such as culture and biochemical tests, for detecting and identifying bacterial pathogens. Among these methods, the commercial Rapid ID32 Strep system is widely used for identifying *L. garvieae* isolates. With this commercial identification system, the strain of *L. petauri* was identified as *Enterococcus gallinarum*, exhibiting a biochemical profile slightly different to that observed in *L. petauri* trout isolates [8]. As expected, MALDI-TOF was also unable to correctly identify the *L. petauri* strain. These data corroborate the difficulty of the accurate identification of this species by using methods commonly applied in the routine of diagnostic laboratories. Although *L. garvieae* and *L. petauri* cannot be accurately identified with the Rapid ID32 Strep strips, both species exhibit some biochemical distinctions that could represent phenotypic indicators for their differentiation. Thus, the *L. petauri* isolate hydrolyzed hippurate and produced acid from sucrose and tagatose, biochemical traits that have been proposed for the recognition of *L. petauri* isolates, while isolates with negative reactions to these tests correspond to *L. garvieae* [8]. The genetic characteristics of the *L. petauri* strain isolated from a wild *L. gibbosus* in Italy is in line with previously documented data for strains found in the Mediterranean region [8]. Considering virulence factors in this strain, only *hly2* was found in the absence of symptoms. However, Teker et al. [22] reported for *L. garvieae* that *hly1* and *hly2* appeared to directly participate in pathogenesis, while *hly3* might not be responsible for the strains' virulence. The role of different hemolysins in *L. petauri* remains unknown, but the fact that the *L. petauri* isolate did not carry the *hly1* gene could explain why *L. petauri* in *Lepomis gibbosus* was not able to spread outside the eye, unlike that usually reported for *L. garvieae* infections. The capsule gene cluster was also negative. Many *L. garvieae* strains without capsules have been reported as highly virulent in natural outbreaks, suggesting that the capsule could enhance pathogenicity but is not essential.

Generally, the *L. petauri* strain isolated here was found to be resistant to several antibiotics since it was sensitive only to ampicillin, amoxicillin, florfenicol, and G-penicillin. Multiple resistance is frequently encountered, referring to the occurrence of resistance to more than one chemotherapeutic agent in one isolate. The spread of antibiotic resistance genes in bacterial populations is aided by various mechanisms of horizontal gene transfer, of which plasmid-mediated transfer is the most widely documented in streptococcal fish pathogens. These results were found in previous studies where ampicillin was the most active agent against *L. garvieae* strains [27].

The transition of bacteria from wild fish to farmed fish can occur rapidly. Thus, the initial detection of *L. petauri* in a wild fish species raises concerns for national trout farms. It could represent an additional risk to the country's aquaculture, potentially infiltrating production activities and exacerbating the already delicate health situation, particularly in lowland production systems where water temperatures remain high from spring to late autumn.

Furthermore, the passage of pathogens from farmed fish to wild fish poses a further problem with respect to the biodiversity of naïve fish populations in lacustrine and fluvial environments [25,28].

A monitoring campaign to know the real prevalence of this pathogen could be useful to estimate the risk represented for wild and farmed fish species.

This study represents the first description of *L. petauri* in a wild fish (*L. gibbosus*) within Lake Candia, a natural lake in Northwestern Italy. This discovery is significant for several reasons:

- The identification of *L. petauri* in a new host species expands our knowledge of its host tropism;
- *L. petauri* could have implications for public health, either directly or indirectly. While *L. petauri* may not pose an immediate threat to human health, understanding its distribution and hosts can be relevant for managing and monitoring potential risks, especially if its zoonotic potential is confirmed [10].

5. Conclusions

With this report, a possible extremely complicated scenario emerges that could occur quickly in the aquaculture sector, as has happened in Turkey, Greece, and Spain where the new species has practically replaced the old pathogen *L. garvieae*. It is therefore essential to conduct new epidemiological studies at a national level to monitor any changes in fish sanitary status in real time, extending this study also to wild fish fauna and fish-eating birds and in water that can act as a reservoir for the pathogen.

Many pathogenic aspects relating to this bacterial species and the immune response induced in the host remain to be understood and therefore studies related to these topics should be carried out aimed at developing prophylactic measures to control disease spreading.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9040117/s1, Table S1: A summary of the interpretive categories and breakpoints (mm) (streptococci species were used). Interpretative criteria outlined by the Clinical and Laboratory Standards Institute (CLSI) and the studies on *L. petauri* conducted by Cataõ Egger and colleagues in 2022 [10]. Figure S1: Amplification of hemolysin genes. 1—Hly1 sample; 2—Hly1 PCR negative control; 3—Hly1 PCR positive control; 4—Hly2 sample; 5—Hly2 PCR negative control; 6—Hly2 PCR positive control; 7—Hly3 sample; 8—Hly3 PCR negative control; 9—Hly3 positive control; L—AmpliSize Molecular Ruler (50–2000 bp Ladder).

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