Received: 14 October 2022 | Revised: 24 April 2023 | Accepted: 29 April 2023

DOI: 10.1002/aah.10188

ARTICLE

Detection and virulence of *Lactococcus garvieae* and *L. petauri* from four lakes in southern California

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Abstract

Objective: The first objective of the study aimed to detect the presence of *Lactococcus petauri*, *L. garvieae*, and *L. formosensis* in fish ($n = 359$) and environmental ($n = 161$) samples from four lakes near an affected fish farm in California during an outbreak in 2020. The second objective was to compare the virulence of the *Lactococcus* spp. in Rainbow Trout *Oncorhynchus mykiss* and Largemouth Bass *Micropterus salmoides*.

Methods: Standard bacterial culture methods were used to isolate *Lactococcus* spp. from brain and posterior kidney of sampled fish from the four lakes. Quantitative PCR (qPCR) was utilized to detect *Lactococcus* spp. DNA in fish tissues and environmental samples from the four lakes. Laboratory controlled challenges were conducted by injecting fish intracoelomically with representative isolates of *L. petauri* ($n = 17$), *L. garvieae* ($n = 2$), or *L. formosensis* ($n = 4$), and monitored for 14 days postchallenge (dpc).

Result: *Lactococcus garvieae* was isolated from the brains of two Largemouth Bass in one of the lakes. *Lactococcus* spp. were detected in 14 fish (8 Bluegills *Lepomis macrochirus* and 6 Largemouth Bass) from 3 out of the 4 lakes using a qPCR assay. Of the collected environmental samples, all 4 lakes tested positive for *Lactococcus* spp. in the soil samples, while 2 of the 4 lakes tested positive in the water samples through qPCR. Challenged Largemouth Bass did not show any signs of infection postinjection throughout the challenge period. Rainbow Trout infected with *L. petauri* showed clinical signs within 3 dpc and presented a significantly higher cumulative mortality (62.4%; $p < 0.0001$) at 14 dpc when compared to *L. garvieae* (0%) and *L. formosensis* (7.5%) treatments.

Conclusion: The study suggests that qPCR can be used for environmental DNA monitoring of *Lactococcus* spp. and demonstrates virulence diversity between the etiological agents of piscine lactococcosis.

KEYWORDS

emerging, lactococcosis, salmonids

INTRODUCTION

Piscine lactococcosis is an acute hemorrhagic bacterial disease that affects freshwater and marine fish species in Europe, South America, North America, and Asia (Vendrell et al. 2006; Gibello et al. 2016; Fukushima et al. 2017; Ortega et al. 2020; Shahin et al. 2022). The causative agent of piscine lactococcosis was historically considered to be *Lactococcus garvieae*, an opportunistically zoonotic, gram-positive bacterium responsible for costly outbreaks of high mortality in wild and cultured fish populations worldwide (Colorni et al. 2003; Algöet et al. 2009). *Lactococcus* spp. were originally assigned to the *Streptococcus* genus but have been reclassified over the years through the use of highly sensitive and informative diagnostic tools (Eldar and Ghittino 1999; Vendrell et al. 2006). Several important species of the genus *Lactococcus* with known and emerging clinical consequences in fish have been identified, including *L. garvieae*, *L. plantarum*, *L. piscium*, and *L. raffinolactis* (Vendrell et al. 2006; Soltani et al. 2021). In 2014, *L. formosensis* sp. nov. was isolated from a fermented broccoli stem and identified as a new species in the genus *Lactococcus* (Chen et al. 2014). This was followed by the description of *L. petauri* from a sugar glider *Petaurus breviceps* lesion (Goodman et al. 2017; Kotzamanidis et al. 2020). Both novel strains share a significant portion of their genome with *L. garvieae* and have also been recently identified as etiologic agents of piscine lactococcosis in various species of fish, such as Barramundi *Lates calcarifer*, Rainbow Trout *Oncorhynchus mykiss*, and catfish (Siluriformes), in Canada, Mexico, and the United States (Heckman et al. 2022).

Clinical manifestation of infections caused by *L. garvieae*, *L. petauri*, or *L. formosensis* is similar and can include acute hemorrhagic septicemia, erratic swimming, lethargy, exophthalmia, anorexia, skin pigmentation changes, and moderate to high mortality in affected systems (Vendrell et al. 2006; Shahin et al. 2021). Additionally, commonly used diagnostic methods, such as biochemical identification kits, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and published molecular methods, cannot discern among the three species or have not been fully validated (Martinovic et al. 2021; Heckman et al. 2022; Egger et al. 2023). Currently, the most appropriate methods to differentiate among these three *Lactococcus* spp. are via DNA gyrase subunit B (*gyrB*) sequence, multilocus sequence analysis, or whole-genome sequencing analysis (Heckman et al. 2022).

An increased risk of transmission and a higher virulence of piscine lactococcosis in salmonids occur in water temperatures higher than 15°C (Algöet et al. 2009; Shahin et al. 2021). Horizontal transmission occurs primarily through contaminated waters and contact with infected or asymptomatic fish (Muzquiz et al. 1999; Eyngor

Impact statement

Salmonids are a keystone species in the Pacific Northwest, given their key role in nutrient cycling and as a vital food source for humans and animals. Gaining a better understanding of the distribution, prevalence, virulence, and diversity of emerging pathogens in wild and captive fish is important for the development of containment and preventative protocols.

et al. 2004). Subclinically infected Rainbow Trout can carry *L. garvieae* for months, with one study detecting *L. garvieae* for 1 year after an outbreak, and can transmit disease to other species of susceptible fish if conditions are conducive (Zlotkin et al. 1998; Algöet et al. 2009). Mechanical transmission through migrating birds and other vectors has been reported (Algöet et al. 2009; Shahin et al. 2022) and is a cause for concern over dissemination into noninfected regions. With the increasing demand for aquaculture production, a growing concern for the interactions between farmed and wild fish is emerging (Murray and Peeler 2005; Vendrell et al. 2006; Lafferty et al. 2015; Teixeira Alves and Taylor 2020). There is a risk that wild fish will transmit pathogens to farmed fish, causing economically significant losses to producers (Colorni et al. 2003; Algöet et al. 2009). Additionally, pathogen transfer from farmed fish to wild fish poses a threat to biodiversity and naïve populations, thus endangering wildlife and impacting biodiversity. Aquaculture fish stocks tend to be more susceptible to infectious diseases due to greater animal densities, which cause increased host contact rates and higher stress in some situations (Lafferty et al. 2015).

Lactococcus garvieae, *L. petauri*, and *L. formosensis* have the potential to infect a wide range of hosts, including domestic animals, wildlife, humans, crustaceans, and fish (Colorni et al. 2003; Evans et al. 2006; Vendrell et al. 2006; Gibello et al. 2016). However, the zoonotic potential and fish host specificity of these bacteria have not been fully investigated. The increasing number of *L. garvieae* cases in humans has been linked to the handling and consumption of raw fish and seafood and is particularly concerning for immunocompromised individuals (Gibello et al. 2016).

In the Americas, recent outbreaks of piscine lactococcosis have occurred in at least four states within the United States, as well as in Canada, Mexico, and Brazil (Evans et al. 2009; Ortega et al. 2020; Shahin et al. 2021; Heckman et al. 2022), resulting in significant economic losses and high mortality rates among cultured fish (Rowan and Morse 2020). In spring 2020, although initially diagnosed as *L. garvieae*, *L. petauri* was identified for the first time in California as the etiological agent of piscine lactococcosis at four different trout aquaculture facilities, resulting in the euthanasia of 3.2 million trout (Rowan and Morse 2020; Shahin et al. 2021). The origin of the emergent isolate of *L. petauri* in California is unknown, as is the prevalence of this bacterium or the closely related *L. garvieae* and *L. formosensis* in wild fish. Furthermore, isolates from the 2020 outbreak in California proved to be highly virulent for Rainbow Trout in laboratory-controlled challenges and were difficult to treat in natural outbreaks (Shahin et al. 2021; E. Littman and colleagues, unpublished). To understand the genetic diversity of the agents of piscine lactococcosis in the North American outbreaks, 23 isolates recently recovered from diseased fish in four different U.S. states as well as Mexico and Canada over the past 5 years were evaluated through multilocus sequence analysis and whole-genome sequencing to identify them as *L. garvieae*, *L. petauri*, or *L. formosensis* (Heckman et al. 2022). Fourteen isolates from previous U.S. outbreaks, including the California outbreaks originally identified as *L. garvieae* through quantitative polymerase chain reaction (qPCR; Shahin et al. 2022), have recently been recharacterized as *L. petauri* through whole-genome sequencing (Heckman et al. 2022). Still, the presence of *Lactococcus* spp. in the environment, particularly in the areas surrounding affected fish farms, is unknown.

In this study, we first aimed to conduct surveillance of the etiological agents of piscine lactococcosis—*L. garvieae*, *L. petauri*, and *L. formosensis*—in the ecosystems near an affected hatchery to better understand the distribution of these pathogens in surrounding aquatic environments and hosts during an outbreak of the disease. Furthermore, our second objective was to compare the virulence of North American isolates of *L. garvieae*, *L. petauri*, and *L. formosensis* in two relevant fish species to gain insights into host susceptibility and the pathogenicity of individual isolates.

METHODS

Detection of *Lactococcus* spp. in environmental samples and wild fish from four lakes in southern California

In September 2020, the California Department of Fish and Wildlife (CDFW) collected a total of 359 wild fish from four lakes located near the Mojave River Hatchery (Victorville, California), a facility that was affected by the *L. petauri* outbreaks during April–September 2020 (Rowan and Morse 2020; Shahin et al. 2021). The four lakes sampled were Victor Valley College ($n = 78$), Spring Valley ($n = 77$), Jess Ranch ($n = 62$), and Horseshoe ($n = 142$) lakes (Figure 1; Table 1). The eight species of fish obtained included Largemouth Bass (LMB; $n = 160$), Channel Catfish ($n = 8$), Black Crappie ($n = 3$), Green Sunfish ($n = 4$), Bluegill (BG; $n = 139$), Common Carp ($n = 20$), Threadfin Shad ($n = 13$), and Prickly Sculpin ($n = 12$; Table 1).

Additionally, soil and water samples ($n = 161$) were collected from five randomly selected locations at each lake for further environmental DNA (eDNA) analysis as described by Richey et al. (2018). Triplicate samples from each location were collected. Fish and environmental samples, refrigerated in plastic coolers with icepacks, were transferred to the University of California–Davis for further analysis.

Necropsies and a standardized health assessment were completed for each collected fish at the University of California–Davis. Fish were examined for external and internal gross changes. Morphometric data were documented for each individual, including total length (cm), weight (g), and sex (if determinable). Brain (Br) and posterior kidney (PK) swabs were collected from each specimen and inoculated onto tryptic soy agar supplemented with 5% sheep blood (blood agar) plates (Biological Media Services), which were incubated for 72 h at 30°C for further evaluation of *Lactococcus* spp. prevalence. Pure bacterial colonies were identified using Gram staining, catalase tests (3% H_2O_2 volume/volume), and qPCR, as described below. Tissue samples of PK, Br, spleen (Sp), internal organs (IO), and/or liver were aseptically collected from fish larger than 1 g and were stored at −80°C for further molecular analysis. Fish under 1 g were processed as a whole.

FIGURE 1 Map depicting the spatial distribution of the four sampled lakes located near the affected hatchery (Mojave River Hatchery, Victorville, California, United States). The Mojave River Hatchery, Spring Valley Lake, and Horseshoe Lake are connected through a water canal that exits the hatchery and enters Spring Valley Lake. Spring Valley Lake and Mojave Narrows Regional Park, which includes Horseshoe Lake, are connected through an additional canal. Jess Ranch Lake is isolated, with no known connections. Victor Valley College Lake has no known outlets or connections.

Total DNA from tissue samples (PK, Br, Sp, IO, liver, or whole body in the case of small fish) and bacteria was extracted using DNeasy Blood and Tissue Kits (Qiagen) following the manufacturer's instructions. Total DNA extractions of water and soil samples were performed using the DNeasy PowerWater and DNeasy PowerSoil Kits (Qiagen), respectively, following previously published protocols (Richey et al. 2018). Concentrations of genomic DNA (gDNA) and eDNA were measured on a NanoDrop UV–Vis spectrophotometer (Fisher Scientific). Extracted gDNA and eDNA were stored at −20°C until analysis by qPCR.

The presence of piscine pathogenic *Lactococcus* was investigated in gDNA and eDNA using a TaqMan-based qPCR assay (Shahin et al. 2022). For all tests, a cycle threshold (C_t) value less than 36 in all three replicate wells was considered a positive detection, whereas C_t values of 36–40 in at least two of the three replicate wells were considered suspect or weakly positive.

TABLE 1 Prevalence of *Lactococcus* spp. (*L. garvieae*, *L. petauri*, or *L. formosensis*) in the fish species submitted for testing from four lakes located near the affected hatchery (Mojave River Hatchery, Victorville, California, United States).

Species	Horseshoe Lake	Jess Ranch Lake	Spring Valley Lake	Victor Valley College Lake	Total
Largemouth Bass *Micropterus salmoides*	2/52[a]	35	32	4/41[a]	6/160[a]
Channel Catfish *Ictalurus punctatus*	2	1	5	0	8
Black Crappie *Pomoxis nigromaculatus*	1	0	1	1	3
Bluegill *Lepomis macrochirus*	7/86[a]	17	1/12[a]	24	8/139[a]
Common Carp *Cyprinus carpio*	0	9	11	0	20
Threadfin Shad *Dorosoma petenense*	0	0	13	0	13
Prickly Sculpin *Cottus asper*	0	0	0	12	12
Green Sunfish *Lepomis cyanellus*	1	0	3	0	4
Total number of fish	142	62	77	78	359
Prevalence (%)	6.3	0	1.3	5.1	3.9

[a]Fish sample that tested positive for *Lactococcus* spp. by quantitative polymerase chain reaction.

Comparison of qPCR results between the different locations and sampling groups was performed to determine the bacterial prevalence for the study addressing objective 1. Through box-and-whisker plots for each lake, we correlated the percentage of positive fish samples to the percentage of positive environmental samples by their respective C_t distributions using GraphPad Prism version 8.3.0. Exact binomial confidence intervals were calculated for the percentage of positive *Lactococcus* spp. results from each lake. Due to the small sample size of four lakes, a limited spatial analysis was assessed by the shortest distance to the Mojave River Hatchery and its outflows. Frequencies of positive samples (soil, water, and tissue) among the four lakes were analyzed through Fisher's exact test using GraphPad Prism version 8.3.0. Prevalence was calculated using the following formula:

$$\frac{\text{Number of positive samples}}{\text{Total number of samples per lake}}.$$

Virulence of *Lactococcus garvieae*, *L. formosensis*, and *L. petauri* isolates in Rainbow Trout and Largemouth Bass

To investigate the virulence of the recovered *Lactococcus* spp. isolates recovered from outbreaks of disease and surveillance studies (objective 1), we conducted in vivo laboratory-controlled challenges with Rainbow Trout and LMB. Rainbow Trout were used because most of the outbreaks reported in the United States have occurred in this species. Largemouth Bass were also used, as the two *L. garvieae* isolates obtained in the surveillance study were recovered from feral LMB. Prior to in vivo challenges, all methods were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California–Davis (Protocol 22321). Rainbow Trout (average weight = 9.6 g) and LMB (average weight = 25 g) used in this experiment were obtained from sources with no history of piscine lactococcosis. Subsets of fish ($n = 10$ per species) were euthanized and confirmed to be free of clinical signs, lesions, and culturable bacteria by complete necropsy and PK culture on blood agar prior to challenge.

All fish were housed at the Center for Aquatic Biology and Aquaculture at the University of California–Davis in a biosecurity level-2 research facility approved by CDFW to conduct laboratory-controlled infectious disease challenges in fish. Fish were acclimatized at the facility for at least 1 month prior to the study. Fish were challenged in 18-L flow-through tanks supplied with fresh well water at a flow rate of 0.5 L/min and supplemental aeration, and they were allowed to acclimate to the tanks for at least 1 week prior to bacterial challenge. Water temperature was monitored with digital thermometers daily and maintained at 18 ± 1°C. Fish were fed 1% of their body weight per day with a commercial pelleted diet (Skretting).

Lactococcus garvieae, *L. formosensis*, and *L. petauri* isolates (Table 2) were inoculated onto blood agar plates and incubated at 30°C for 24–48 h. Bacteria were suspended in 5 mL of sterile 1× phosphate-buffered saline (PBS) to achieve a dilution corresponding to 0.5 McFarland turbidity standard (~10^8 colony-forming units [CFU]/mL). Confirmation of bacterial dilution (CFU/mL) was performed by a 6 × 6 drop plate method (Chen et al. 2014) with 10 µL of each 10-fold dilution on blood agar after incubation at 30°C for 24–48 h.

Prior to injection, Rainbow Trout were anesthetized with sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Syndel) at a dose of 50 mg/L, whereas LMB were anesthetized with an MS-222 dose of 100 mg/L. Anesthetized fish were injected with 0.1 mL of either sterile 1× PBS (for negative control groups) or bacterial suspension by intracoelomic injection. Each bacterial isolate was inoculated into 10 Rainbow Trout and 10 LMB kept in individual tanks ($n = 10$ fish/tank per isolate). After exposure, fish were monitored twice daily for the onset of clinical signs, moribundity, and mortalities for 14 days. Moribund fish were defined as having (1) one severe clinical sign, (2) two or more clinical signs at any one time, or (3) a single clinical sign lasting more than 24 h. Moribund fish were euthanized with a 500-mg/L dose of buffered MS-222 in accordance with American Veterinary Medical Association guidelines for euthanasia (Leary et al. 2020).

For each experimental tank, the first five mortalities and three to five survivors at the end of each trial were screened for the presence of *Lactococcus* spp. using culture methods. Aseptic swabs of PK and Br were inoculated onto blood agar and incubated at 30°C for 24–48 h. Presumptive bacterial identification at the genus level was made with Gram staining and the catalase test. Gram-positive cocci that were catalase negative were interpreted as *Lactococcus* spp.

Survival curve analysis and statistics of challenged Rainbow Trout and LMB were performed using GraphPad Prism v.8.3.0. Statistical significance was determined by Log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests. One-way analysis of variance (ANOVA) was used to compare bacterial persistence among survivors. If the difference was $p < 0.05$, it was statistically significant.

TABLE 2 Comparison of Rainbow Trout and Largemouth Bass survival and bacterial persistence following challenge with *Lactococcus garvieae*, *L. petauri*, or *L. formosensis*. *Lactococcus* spp. were identified based on DNA gyrase subunit B sequence comparisons (Heckman et al. 2022).

				Rainbow Trout			Largemouth Bass		
Species	**Strain**	**Reference**	**Origin**	**Survival (%)**	**Bacterial persistence (%)**	**Cumulative mortality by species (%)**	**Survival (%)**	**Bacterial persistence (%)**	**Cumulative mortality by species (%)**
L. petauri	MOJ1	Shahin et al. (2021)	USA	20	0	62.4	100	0	0
	JR1	Shahin et al. (2021)	USA	40	50		100	10	
	FS1	Shahin et al. (2021)	USA	40	100		100	0	
	BR1	Shahin et al. (2021)	USA	20	0		100	0	
	CDFW 0913 Hotcreek_C_3_6_kid	Heckman et al. (2022)	USA	30	67		100	0	
	LP18-Lg12 (G1)	Ortega et al. (2020)	Mexico	20	100		100	0	
	RC17-Lg01 (G2)	Ortega et al. (2020)	Mexico	10	100		100	0	
	Mic16-09 (G3)	Ortega et al. (2020)	Mexico	40	100		100	0	
	AC16-Lg13 (G4)	Ortega et al. (2020)	Mexico	0	N/A		100	0	
	R21-69 (HC)	Heckman et al. (2022)	USA	20	100		100	0	
	S69-A	Heckman et al. (2022)	USA	100	0		100	0	
	M17100408 FF4i7	Heckman et al. (2022)	USA	40	75		100	0	
	LG-PAQ102015-99 (LG-WV1)	Nelson et al. (2016)	USA	0	N/A		100	0	
	R21-77 Canada	Heckman et al. (2022)	Canada	10	90		100	10	
	Isol 5-Hyb Catfish RW-S6 May (R21-74)	Heckman et al. (2022)	USA	90	90		100	0	
	Isol 1-Hyb Catfish RW-S6 July (R21-74)	Heckman et al. (2022)	USA	60	10		100	0	
	M18012501 FF2g6	Heckman et al. (2022)	USA	100	60		100	0	
L. garvieae	VC11	This study	USA	100	80	0	100	0	0
	VC13	This study	USA	100	75		100	0	
L. formosensis	M20011502 FF20a2	Heckman et al. (2022)	USA	100	0	7.5	100	0	0
	M04020401 EESe5	Heckman et al. (2022)	USA	100	60		100	0	
	M03102409 EESd7	Heckman et al. (2022)	USA	70	20		100	0	
	LG-PAQ102015-208 (LG-WV2)	Heckman et al. (2022)	USA	100	40		100	0	

RESULTS

Detection of *Lactococcus* spp. in environmental samples and wild fish from four lakes in southern California

Lactococcus garvieae was isolated from Br tissue in 2 of 41 LMB sampled from Victor Valley College Lake. No culturable *L. petauri* or *L. formosensis* was recovered from PK or Br swabs. No gross external or internal lesions were observed during the necropsies of the fish. In a few cases, metazoan parasites were visualized in the coelomic cavity. Fourteen of the 359 fish sampled tested positive for *L. garvieae*, *L. petauri*, or *L. formosensis* (collectively referred to hereafter as "*Lactococcus* spp.") via qPCR analysis (Table 3). Of the eight fish species collected for analysis, *Lactococcus* spp. were detected only in BG and LMB through qPCR (Table 3). Three of the four lakes contained fish that tested positive for the bacteria via qPCR, with the highest fish-level prevalence observed at Horseshoe Lake (6.3%), followed by Victor Valley College Lake (5.1%; Table 1); however, there was no statistical difference in the prevalence of positive tissue samples between these two sites ($p = 0.7158$). Fish collected from Jess Ranch Lake did not test positive for *Lactococcus* spp. through qPCR or bacterial isolation. Two of 52 LMB from Horseshoe Lake and 4 of 41 LMB from Victor Valley College Lake had confirmed traces ($C_t < 40$) of *Lactococcus* spp. Seven of 86 BG from Horseshoe Lake and 1 of 12 BG from Spring Valley Lake showed positive detection of *Lactococcus* spp. by qPCR. The IO of BG from Horseshoe Lake had the highest amounts of gDNA, with 2.55 log genome equivalents (GE) µg/gDNA (Table 3). The prevalence of *Lactococcus* spp. was highest in Horseshoe Lake (6.3%), primarily in the sampled BG population, which had the overall highest prevalence among the eight fish species (Table 3). In Spring Valley Lake, 1 of 12 BG was positive for *Lactococcus* spp. in a PK sample. Four of 41 LMB from Victor Valley College Lake had positive samples from Br tissue detected through qPCR. Most fish had two tissue samples taken at necropsy; however, only one tissue sample from each fish that tested positive had detectable traces of pathogen through qPCR. Internal organs collected from fish less than 1 g had the highest prevalence of *Lactococcus* spp. (50%), followed by the Br tissue samples collected from fish ranging from 0.4 to 88 g (35.7%).

Laboratory-controlled challenges in Largemouth Bass and Rainbow Trout fingerlings

No clinical signs of piscine lactococcosis or mortalities were observed in any of the challenged LMB (Table 2). Two of the 240 challenged LMB were positive for *L. petauri* through bacterial isolation and qPCR at the end of the challenge. The positive fish were infected with the *L. petauri* JR1 (U.S. origin) and R21-77 (Canadian origin) isolates (Table 2).

Significantly greater disease severity and mortality associated with piscine lactococcosis were observed in Rainbow Trout groups challenged with distinct isolates of *L. petauri* in comparison with Rainbow Trout groups that were exposed to the two other bacterial species ($p < 0.0001$). Rainbow Trout exposed to the *L. petauri* strains ($n = 17$) showed an average survival of 37.6% at 14 days postchallenge (dpc), while only one of the *L. formosensis* strains (M03102409 EESd7) resulted in the mortality of three fish (Table 2). For challenges that used the *L. garvieae* group of isolates ($n = 2$), 100% of exposed Rainbow Trout survived (Table 2). The onset of mortality began at 3 dpc with the majority of *L. petauri* isolates, while the first mortality for an *L. formosensis* isolate was recorded at 6 dpc. *Lactococcus petauri*, *L. garvieae*, and *L. formosensis* were reisolated from the PK of 62.8, 77.5, and 30.0% of surviving fish, respectively (Table 2). There was no significant difference among the bacterial species in terms of persistence in the surviving fish ($p = 0.2487$). All negative controls remained uninfected, and no mortality of controls occurred throughout the study.

DISCUSSION

After the initial diagnosis of piscine lactococcosis in California (Shahin et al. 2021) and realization that this disease might be caused by different bacterial species, many questions arose regarding the epidemiology and virulence of *L. garvieae*, *L. petauri*, and *L. formosensis* in the western United States. Our first aim was to gain a better understanding of the prevalence of the potential pathogens in natural lakes surrounding an affected fish hatchery through fish and environmental samples that were evaluated using conventional microbiological methods and eDNA detection.

Lactococcus spp. were detected in fish tissues, water, and soil in all lakes surrounding the Mojave River Hatchery. Interestingly, the two isolates recovered from LMB were genetically distinct from those recovered during the 2020 outbreaks, identified as *L. garvieae* rather than *L. petauri*, confirming genetic heterogeneity in the region. The host range of *L. garvieae* has been reported to be "wide," with outbreaks occurring in the Striped Mullet *Mugil cephalus*, Wels *Silurus glanis*, giant freshwater prawn *Macrobrachium rosenbergii*, and Nile Tilapia *Oreochromis niloticus* (Vendrell et al. 2006; Meyburgh et al. 2017). *Lactococcus* spp. were detected in only two of the feral fish species evaluated in the first study (BG and

TABLE 3 Detection of piscine *Lactococcus* spp. (*L. garvieae*, *L. petauri*, or *L. formosensis*) in feral fish through quantitative polymerase chain reaction. The fish species is indicated by the identifier (Bluegill [BG] or Largemouth Bass [LMB]). Fish under 1 g were processed as a whole due to the possibility of contamination during harvest of organs. Br, brain; IO, internal organs; PK, posterior kidney.

Identifier	Tissue	Lake	Weight (g)	Length (cm)	Load[a]
BG80	Br	Horseshoe	<0.4	2.0	0.20
BG29	IO	Horseshoe	0.6	4.0	0.55
BG33	IO	Horseshoe	0.8	4.0	2.40
BG43	IO	Horseshoe	<0.4	3.5	0.93
BG55	IO	Horseshoe	<0.4	3.5	2.55
BG56	IO	Horseshoe	<0.4	3.0	1.67
BG59	Body/tail	Horseshoe	<0.4	2.5	1.82
LMB50	IO	Horseshoe	0.5	8.0	1.66
LMB51	IO	Horseshoe	0.5	9.0	2.09
BG31	PK	Spring Valley	7.0	9.0	1.13
LMB11	Br	Victor Valley College	39.4	18.5	2.09
LMB36	Br	Victor Valley College	0.6	5.0	0.56
LMB5	Br	Victor Valley College	88.0	19.5	1.79
LMB8	Br	Victor Valley College	49.8	15.5	1.87

[a]Load is presented as log *Lactococcus* spp. genome equivalents per microgram of DNA.

LMB), suggesting some host association to the bacteria. Although salmonids, especially Rainbow Trout, have been proposed as one of the main host targets of *L. garvieae* (Vendrell et al. 2006) and *L. petauri*, no cases have been reported in BG or LMB. Outbreaks in wild fish populations can be devastating for ecosystems and are likely to become more frequent as climate change creates a more favorable environment for the spread of pathogens (Krkošek 2017). Wild fish can also pose a "carrier" host risk, thus leading to transmission of the pathogen during conducive periods, and can be a potential source of infection to naïve populations of cultured fish (Vendrell et al. 2006; Algöet et al. 2009; Nelson et al. 2016; Ortega et al. 2020; Shahin et al. 2021). In the current study, no evidence of morbidity or mortality was found in any of the wild fish sampled. No wild salmonids were collected in this study; as such, it is not possible to confirm whether any wild salmonid species near the hatchery was infected with the pathogen.

The presence of salmonids in the sampled lakes is suspected, as three of the lakes (Jess Ranch, Spring Valley, and Horseshoe lakes) are stocked yearly by private and state programs in the winter (CDFW, https://wildlife.ca.gov/Fishing/Hatcheries); however, the salmonid population is known to fluctuate seasonally, and many fish do not survive, particularly during the warmer months. Since this disease is known to have a direct association with environmental temperatures greater than 15°C, the time of year at which surveillance studies are conducted may be critical (Eyngor et al. 2004; Soltani et al. 2008; Pastorino et al. 2019).

The samples of IO (from fish <1 g) and Br tissue collected from the wild fish had the highest prevalence of *Lactococcus* spp. detected through qPCR. The Br is one of the most common targets for detection of closely related *Streptococcus* spp. due to their ability to potentially cross the blood–Br barrier and multiply in the Br tissues (Vendrell et al. 2006; Pradeep et al. 2016). Piscine lactococcosis will often present as septicemia in fish; thus, the kidney and Sp are also regular sites for detection of infection due to the bacteria's ability to disseminate into secondary lymphoid organs (Vendrell et al. 2006; Ortega et al. 2020). *Lactococcus garvieae* can also be detected in the blood, eyes, liver, and intestines, with coinciding lesions in these organs as well (Eldar and Ghittino 1999; Shima et al. 2006; Vendrell et al. 2006; Pradeep et al. 2016). We chose to sample multiple organs, such as Br and PK, from the wild fish due to a lack of knowledge regarding the pathogenicity of *Lactococcus* spp. in BG, LMB, and other fish sampled in this study.

Direct and indirect horizontal transmission routes have been hypothesized for *L. garvieae* (Vela et al. 2000; Vendrell et al. 2006; Pastorino et al. 2019). Besides transmission by clinically and subclinically infected fish hosts, transmission through contaminated water, soil, and sand or through fecal contamination of environments by piscivorous birds has been proposed (Vendrell et al. 2006; Algöet et al. 2009; Eraclio et al. 2019; Shahin et al. 2022). In a recent study, vertical transmission of streptococci was suggested in 10-day-old tilapia *Oreochromis* spp. (Pradeep et al. 2016), indicating a need to further the understanding of the transmission abilities of *Lactococcus* spp. Moreover, *L. garvieae* can persist in a wide range of environments without the fish host and can cause infection in other vertebrates and invertebrate animals (Klijn et al. 1995;

Aguado-Urda and Cutulli 2010; Gibello et al. 2016; Shahin et al. 2021). Through the formation of resistant biofilms, *L. petauri* was recently shown to have the potential to persist in the environment unless treated with specific chemicals (Shahin et al. 2021).

The utilization of molecular methods, such as qPCR, in the detection and quantification of eDNA allowed us to gain a better understanding of the prevalence of *L. garvieae*, *L. petauri*, and *L. formosensis* in the surrounding environments during and after an outbreak. The prevalence of these *Lactococcus* spp. was higher in the soil samples, with all four lakes testing positive, while two of the four lakes displayed positive water samples. Three of the four lakes had an overlap of fish tissue and environmental (soil or water) samples that tested positive for *Lactococcus* spp. (Figure 2). Horseshoe Lake had detectable traces of *Lactococcus* spp. gDNA in fish samples and environmental samples (soil and water). Spring Valley and Victor Valley College lake had soil and tissue samples that contained the amplified eDNA sequences; however, *Lactococcus* spp. were not detected in the water samples. Despite the high prevalence of *Lactococcus* spp. eDNA detection in the soil and water samples from Jess Ranch Lake (40% and 100%, respectively), there was no positive detection of infection from the sampled wild fish. Our results indicate that eDNA is a useful tool for surveillance of infectious agents in the environment, particularly in the soil, and that this qPCR test is useful for detecting *Lactococcus* spp. bacterial loads in the environment.

Laboratory-controlled challenge studies confirmed a greater susceptibility of Rainbow Trout to piscine lactococcosis, particularly to *L. petauri* infections. Previous studies have also shown high virulence of perceived *L. garvieae* isolates in laboratory-controlled challenges using Rainbow Trout, Gray Mullet, and European Grayling *Thymallus thymallus* infection models (Vendrell et al. 2006). The present study was the first to challenge LMB with isolates of *Lactococcus* spp. and to investigate the pathogenicity of these agents in LMB. Although the LMB showed no clinical signs of morbidity in response to the *Lactococcus* isolates, objective 1 did confirm that they can be carriers of the bacteria and, thus, may pose a threat to susceptible salmonids in conducive conditions.

Due to the recent descriptions of *L. petauri* and *L. formosensis* (Chen et al. 2014; Goodman et al. 2017), the complications with historical misidentification of these species as "*L. garvieae*," and the significant effects of these bacterial species in wild and farmed fish, more research is necessary to understand the epidemiology and pathogenesis of the three *Lactococcus* spp. (Heckman et al. 2022). In addition, thorough genomic studies are needed to correct the assignment of the current and previously recovered piscine lactococcosis species. This will help in the development of accurate and rigorous diagnostics and control measures for this disease in wild systems and aquaculture facilities. Although the high degree of genetic similarity indicates that many shared characteristics are maintained among these

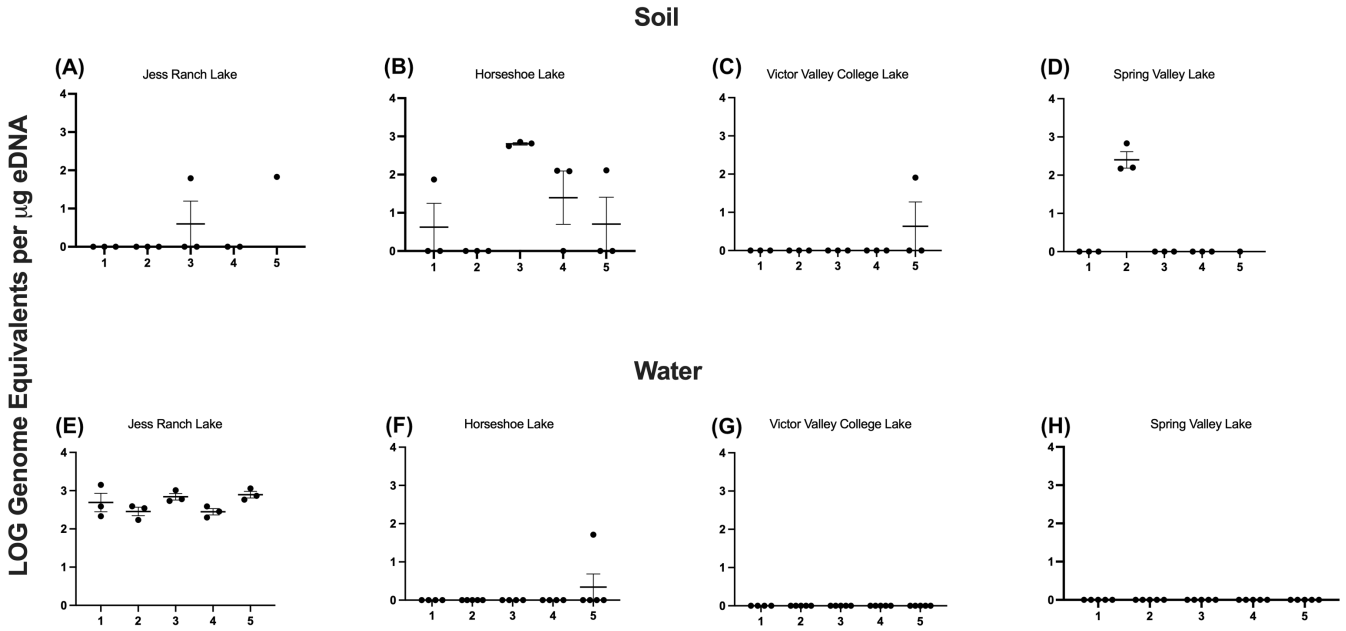

FIGURE 2 *Lactococcus* spp. screening (by quantitative polymerase chain reaction) of (A)–(D) soil or (E)–(H) water samples from four lakes located near the Mojave River Hatchery (Victorville, California): (A), (E) Jess Ranch Lake; (B), (F) Horseshoe Lake; (C), (G) Victor Valley College Lake; and (D), (H) Spring Valley Lake. Results are listed as the log *Lactococcus* spp. genome equivalents (GE) per microgram of total environmental DNA for soil or water collected from each site. Five different sites in each lake were sampled. From each site, three to five different samples were evaluated. Figures are means of GE per microgram ± standard error.

Lactococcus spp., the observed differences in virulence for Rainbow Trout in this study suggest relevant differences for aquaculture.

In conclusion, we have demonstrated the utility of molecular methods in epidemiological analysis of potential fish pathogens in the environment and wild fish following an outbreak in a hatchery. Future studies determining the virulence of *L. petauri* in salmonids and nonsalmonids, as well as epidemiologic surveys to characterize its prevalence, are needed to develop strict control measures and prevent future outbreaks in hatcheries and the local environment.

ACKNOWLEDGMENTS

This project was funded by CDFW. We thank Melissa Garnett and Rick Stout from the Florida Bass Conservation Center for donating the LMB. We wish to also thank the CDFW staff, Quinn Granfors, Jen Hemmert, Yoselin Caliz, Karin Middleton, Brian Wang, Lauren Hall, and Matt Murchison for assistance in sampling the wild fish and collecting environmental samples. R.A.-H. acknowledges the Fondo de Financiamiento de Centros de Investigación en Áreas Prioritarias, Centro Interdisciplinario de Investigación en Acuicultura Sustentable (Project Number 15110027).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

ETHICS STATEMENT

Animal experimental procedures included in this present study were approved by the Institutional Animal Care and Use Committee ethical review committee at University of California–Davis (Protocol 22321).

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