

RESEARCH ARTICLE

Genetic diversity and phenotypic characterization of *Iodobacter limnosediminis* associated with skin lesions in freshwater fish

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Abstract

The relatively unknown genus *Iodobacter* sp. has been repeatedly isolated from skin ulcers and saprolegniosis on freshwater fish in Finland, especially farmed salmonids. Genetic characterization verified that all 23 bacterial isolates studied here belonged to the species *Iodobacter limnosediminis*, previously undescribed from the fish microbiota. Whole-genome pulsed-field gel electrophoresis revealed variability between the *I. limnosediminis* strains, suggesting that they were most likely of environmental origin. Two *I. limnosediminis* strains caused lesions in 27%–53% of brown trout (*Salmo trutta*) injected intramuscularly ($p \leq .05$). The lesions represented moderate to severe tissue damage, but for most fish, the tissues had been repaired by the end of the experiment through the accumulation of fibrocytes and macrophages at the site of the lesion. *I. limnosediminis* was reisolated from some lesions and/or internal organs. Phenotypically and biochemically, *I. limnosediminis* resembles several common bacterial species found in the aquatic environment, as it grows well on several media as whitish medium-sized colonies, is Gram negative and rod-shaped. Here, we characterized *I. limnosediminis* strains with several methods, including MALDI-TOF. This characterization will help in further investigations into the occurrence and possible involvement of *I. limnosediminis* in skin lesions of freshwater fish.

KEYWORDS

freshwater fish, *Iodobacter limnosediminis*, skin lesions

1 | INTRODUCTION

Bacterial colonization of fish surfaces greatly reflects the surrounding aquatic environment, and fish harbour several commensal species. Sometimes, these environmental and commensal bacteria can become opportunistic pathogens. For example, *Flavobacterium* spp. are frequently found in the aquatic environment and on the

surfaces of healthy fish, especially on the gills, but have also been reported as a prevalent cause of fish diseases in freshwater salmonid aquaculture (Austin, 2006; Loch & Faisal, 2015). Indeed, it is not always obvious whether bacteria from the fish microflora are synergistic, commensal or pathogenic and in the carrier stage. The health status and immunity of fish, as well as changes in environmental conditions and bacterial virulence, play a role in

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disease development. Opportunistic bacteria are known to benefit from changing environmental conditions, as they are able to move between hosts and the environment (Brown et al., 2012). For example, virulence can be altered by environmental factors, as demonstrated for the opportunistic fish pathogen *F. columnare* (Kinnula et al., 2017). This has implications for intensive aquaculture in particular, in which the conditions favour the development of virulence in bacterial strains (Sundberg et al., 2016; Wedekind et al., 2010). Recently, there have been reports of emerging bacterial fish diseases caused by bacteria previously described as commensals in fish and their environment, but which have also frequently been isolated from diseased fish and have been described as potentially pathogenic in freshwater fish (Loch & Faisal, 2015; Pękala-Safińska, 2018). Some of these bacterial strains have been experimentally investigated and found to be pathogenic, causing clinical signs and mortality in fish, such as *Kocuria rhizophila* and *Micrococcus luteus* (Pękala et al., 2018), *Shewanella putrefaciens* (Pękala et al., 2015) and novel *Flavobacterium* spp. and *Chryseobacterium* spp. (Bruce et al., 2020).

Iodobacter sp. is not recognized as a fish pathogen or included in the normal microbiota of freshwater fish (Austin, 2006; Austin & Austin, 2016). The genus *Iodobacter* was first established to describe *Iodobacter fluviatilis*, which was isolated from running freshwaters in the UK (Logan, 1989). *Iodobacter* was a monospecific genus for years, and only recently have further species been isolated and characterized, including *Iodobacter limnosediminis* from Arctic lake sediment (Su et al., 2013), *Iodobacter arcticus* from the meltwater sediment of an Arctic glacier (Srinivas et al., 2013) and *Iodobacter ciconiae* from the faeces of an oriental stork (Lee et al., 2019). The genus *Iodobacter* includes psychrophilic freshwater bacteria, and because of such a recent recognition of the species within the genus, there are few descriptions or publications regarding the strains belonging to the genus. However, Carbajal-González et al. (2011) found *Aeromonas* and *Iodobacter* to be common genera associated with saprolegniosis, that is infection caused by oomycetes from the genus *Saprolegnia*, in brown trout (*Salmo trutta*) in Spain. *Iodobacter* sp. was isolated from healthy skin and from saprolegniosis lesions of brown trout, but was not found on the healthy skin of rainbow trout (*Oncorhynchus mykiss*) (Carbajal-Gonzalez et al., 2011). Interestingly, we have repeatedly isolated *I. limnosediminis* from farmed and wild freshwater fish exhibiting clinical signs of skin lesions and/or saprolegniosis and have investigated whether these isolated *I. limnosediminis* strains could be the cause of the observed clinical disease signs. We investigated whether bacteria isolated from diseased fish can be pathogenic to other fish by following Koch's postulates and observing clinical changes in fish in pathological and histological investigations. Furthermore, we characterized the *I. limnosediminis* strains isolated from fish with several genotyping and phenotyping methods to reveal the status of this relatively unknown bacterial species in the aquatic environment and aid in the identification of the bacterium in future research.

2 | MATERIAL AND METHODS

2.1 | Bacterial isolation and culture conditions

Farmed salmonid fish or wild freshwater bream (*Abramis brama*) with clinical signs from freshwater areas of Finland (Table 1) were killed and transported on ice to the laboratory within 24 hr for pathological and bacteriological examination. Wild-caught bream weighed between 370 and 1,700 g and farmed salmonid fish from 16 g to 1,300 g. Salmonid fish samples were taken from farms that had been demonstrated to be free from viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) and infectious salmon anaemia virus (ISAV), as well as bacterial kidney disease caused by *Renibacterium salmoninarum*. For all the studied fish, pathological changes were recorded and samples were taken from altered areas of the skin when present, as well as from the gills and spleen, with a sterile loop and streaked on 1/4 diluted nutrient agar (1/4 NA; 0.075% beef extract, 0.125% peptone, pH 6.8) (Moss et al., 1978) and/or enriched Anacker and Ordal agar (AOA; 0.5% tryptone, 0.05% yeast extract, 0.02% sodium acetate, 0.02% beef extract, pH 7.3–7.4) (Bernardet & Kerouault, 1989). These agars were incubated at 15°C for 7 days, and colonies were inspected every other day. Kidney samples were streaked on a Bacto™ Columbia Blood Agar base (Oxoid) with 5% sheep blood (BA) and incubated at 20°C for 7 days and inspected every other day. If bacterial colonies resembling colonies of *Aeromonas salmonicida* on BA, *Flavobacterium* sp. or *Iodobacter* sp. on 1/4 NA or AOA were observed, they were subcultured on new growth media until pure cultures were obtained and subjected for phenotypic and biochemical analysis. Saprolegniosis was confirmed macroscopically as white or greyish cotton wool-like patches growing on fish. Occasionally, it was further investigated microscopically from skin and gill scrapes to detect saprolegnia-like hyphae and zoosporangia. Pure cultures of *I. limnosediminis* strains isolated from fish and the type strains *I. limnosediminis* DSM 103822^T (=E1^T) and *I. fluviatilis* DSM 3764^T were maintained in Bacto™ Brain Heart Infusion (BHI; BD) broth with 15% glycerol at –70°C. For this study, we selected a total of 23 *I. limnosediminis* isolates from different fish species, locations and clinical disease outbreaks from fish lesions, of which 16 strains were selected for genetic analyses in 2008. Later, we included additional 7 strains (isolated between 2015 and 2018) to genetic analysis in 2019 (Table 1). Finally, morphological, biochemical and MALDI-TOF analyses were done simultaneously for all the 23 *I. limnosediminis* strains revived from the –70°C storage in 2020, and the results of all these studies are presented in this article. The sampling location and year, fish species, tissue of isolation and clinical signs are presented in Table 1.

2.2 | Phenotypic and biochemical characteristics

All the *I. limnosediminis* strains isolated from diseased fish (Table 1), type strains *I. limnosediminis* DSM 103822^T and *I. fluviatilis* DSM

TABLE 1 *Iodobacter limnosediminis* strains isolated from sampled fish, the watercourse in Finland from which the fish were sampled, the year of isolation and host species, gross pathological findings from the sampled fish and the tissue of isolation

<i>Iodobacter limnosediminis</i> strain	Watercourse	Year	Host	Pathological findings	Host tissue	GenBank accession no.
7812/04	Kymijoki	2004	Whitefish	Saprolegniosis	Skin	MW553179
8119/04	Kymijoki	2004	Brown trout	Saprolegniosis	Skin	MW553178
8157/04	Kymijoki	2004	Brown trout	Saprolegniosis	Skin	MW553177
10084/05	Kymijoki	2005	Rainbow trout	Saprolegniosis	Skin	MW553196
10184/05	Kymijoki	2005	Whitefish	Saprolegniosis	Skin	MW553195
1082/05	Vuoksi	2005	Freshwater bream	Skin lesions	Skin	MW553194
3414/05	Vuoksi	2005	Brown trout	Saprolegniosis	Skin	MW553186
4499/05	Kymijoki	2005	Atlantic salmon	Saprolegniosis	Skin	MW553185
4553/05	Kymijoki	2005	Atlantic salmon	Saprolegniosis	Skin	MW553184
762/05	Vuoksi	2005	Freshwater bream	Skin lesion	Skin	MW553180
9659/05	Kymijoki	2005	Rainbow trout	Skin lesions	Skin	MW553175
1284/06	Vuoksi	2006	Freshwater bream	Skin lesions	Skin	MW553191
2726/06	Kymijoki	2006	Brown trout	Saprolegniosis	Gill	MW553190
3286/06	Vuoksi	2006	Freshwater bream	Skin lesions	Skin	MW553189
3353/06	Vuoksi	2006	Rainbow trout	Saprolegniosis	Gill	MW553187
8187/06	Kymijoki	2006	Atlantic salmon	Saprolegniosis	Skin	MW553176
11751/15	Vuoksi	2015	Brown trout	Saprolegniosis	Tail	MW553192
6650/15	Vuoksi	2015	Brown trout	Saprolegniosis	Skin	MW553182
6875/15	Jänisjoki	2015	Brown trout	Saprolegniosis	Skin	MW553181
5745/16	Kymijoki	2016	Brown trout	Saprolegniosis	Eye	MW553183
6114/16	Vuoksi	2016	Brown trout	Saprolegniosis	Skin	MW553197
11210/18	Kymijoki	2018	Brown trout	Saprolegniosis	Skin	MW553193
3297/18	Kymijoki	2018	Brown trout	Saprolegniosis	Fin	MW553188

Note: The 16S rDNA sequence accession no. in GenBank is also provided.

3764^T were cultured on 1/4 NA and incubated 2–3 days at 20°C, and morphology was examined with Gram staining and subjected for biochemical analysis. Biochemical characteristics were observed by noting bubble formation after the addition of 3% (v/v) H₂O₂, and oxidase activity was tested with 1% (w/v) tetramethyl-*p*-phenylenediamine dihydrochloride. Growth at 37°C was examined by streaking pure colonies on BA, and the formation of colonies was observed after 2–7 days of incubation. Glucose oxidation/fermentation and motility were observed in Hugh and Leifson's OF Basal Medium (H-L) (Mac Faddin, 1980) after incubation for 1–3 days at 20°C. Individual test tubes inoculated with *I. limnosediminis* were incubated at 20°C for 2–5 days to assess the assimilation of sucrose and mannitol, hydrolysis of esculin and production of indole (Mac Faddin, 1980). Furthermore, an API 20 NE system (BioMerieux, France) was used according to the manufacturers' instructions to determine the biochemical profiles and identify the studied strains.

The MALDI-TOF database (Bruker) was supplemented with the main spectrum profile (MSP) from *I. limnosediminis* DSM 103822^T according to the manufacturer's instructions (MBT Explorer Module User Manual Revision A, May 2016, Bruker). MALDI-TOF analysis was performed for *I. limnosediminis* strains isolated from fish and for the strains, *I. limnosediminis* DSM 103822^T and *I. fluviatilis* DSM

3764^T. After 3 days of incubation (20°C on 1/4 NA), colony fractions of each strain were spotted twice to the target plate (Bruker MSP 96 target polished steel BC). They were overlaid with 1 µl of matrix solution (50% acetonitrile and 2.5% trifluoroacetic acid solution saturated with α-cyano-4-hydroxycinnamic acid) and allowed to dry. The target plate was inserted into the MALDI-TOF Biotyper smart system (Bruker Daltonik, GmbH), and each spot was analysed with a maximum of 6 × 40 laser shots from different positions of the target spot, with spectrum profiles acquired in automatic mode and using MBT Compass 1.4 software (MBT Compass User Manual Revision E, April 2019, Bruker). The mass spectrometer was calibrated for molecular weights with a range of 3.6–16.9 kDa prior to sample testing using the Bacterial Test Standard (MBT Compass User Manual Revision E, April 2019, Bruker).

2.3 | DNA isolation, PCR amplification and nucleotide sequencing

Year 2008, 16 strains were sequenced in the Department of Biological and Environmental Science at the University of Jyväskylä. For these strains, genomic DNA was isolated with the phenol-chloroform

method (Griffiths et al., 2000). Broad-range bacterial forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer rD1 (5'-AAGGAGGTCATCCAGCC-3') (Lane, 1991) were used to amplify the nearly full-length 16S rRNA gene using Biotools DNA polymerase (Biotools B&M, Spain). PCR fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), primers 27F, rD1 and four internal PCR primers, and an ABI3100 capillary sequencing instrument (Applied Biosystems). Sequencing reactions were compiled using the software package Vector NTI (Invitrogen).

Year 2019, additional *I. limnosediminis* strains 6875/15, 6650/15, 11751/15, 6114/16, 5745/15, 3297/18 and 11210/18 genomic DNA was isolated using a NucleoSpin® Food kit according to the manufacturer's instructions for bacterial DNA isolation (Macherey-Nagel GmbH & Co. KG, Germany). For PCR amplification, 2 µl of template was used. As a negative control, a sample of sterile water was included in DNA isolation. PCR was performed in a total volume of 50 µl containing 200 µM of dNTPs (DyNAzyme, Finnzymes, Finland) and 5 µl of 10-fold concentrated polymerase synthesis buffer, including 20 mM MgCl₂ (Roche FastStart), 20 pmol of each primer and 2.5 U DNA polymerase (FastStart 5 U/yl). The universal primers 8FX (5'-AGAGTTTGATCCTGGCTNAG-3') and 1407R (5'-TGACGGGCGGTGTGTACAA-3') (Angen et al., 1998) were used

to amplify the whole 16S rRNA gene. Thermal cycling was conducted with initial denaturation at 95°C for 5 min, followed by 30 cycles 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and final extension at 72°C for 5 min. Amplification products were run on a 1.5% agarose gel and purified with a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG). The amount and purity of cleaned PCR product were analysed with a NanoDrop spectrophotometer and sequenced using the Sanger method in the DNA Sequencing and Genomics Laboratory at the University of Helsinki.

Partial 16S rRNA gene sequences of *I. limnosediminis* strains isolated from diseased fish were deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov>) (Table 1) and compared with reference strains using the basic local alignment search tool (BLAST). For phylogenetic analysis of the 16S rRNA gene sequences, the type strain *Chromobacterium aquaticum* CC-SEYA-1^T (accession no. NR_044405) was used as an outgroup and consensus sequences were generated from homologous sequence strains of *Iodobacter* genus reference strains *I. limnosediminis* E1^T (accession no. HM031078), *I. ciconiae* H11R3^T (accession no. MK285606), *I. arcticus* M4-9^T (accession no. JN896753), *I. arcticus* M4-16^T (accession no. FM955868) and *I. fluviatilis* ATCC 33051^T (accession no. M22511). All the sequences were aligned with CLUSTAL W, and a phylogenetic tree was constructed with the maximum-likelihood method using the Kimura 2-parameter

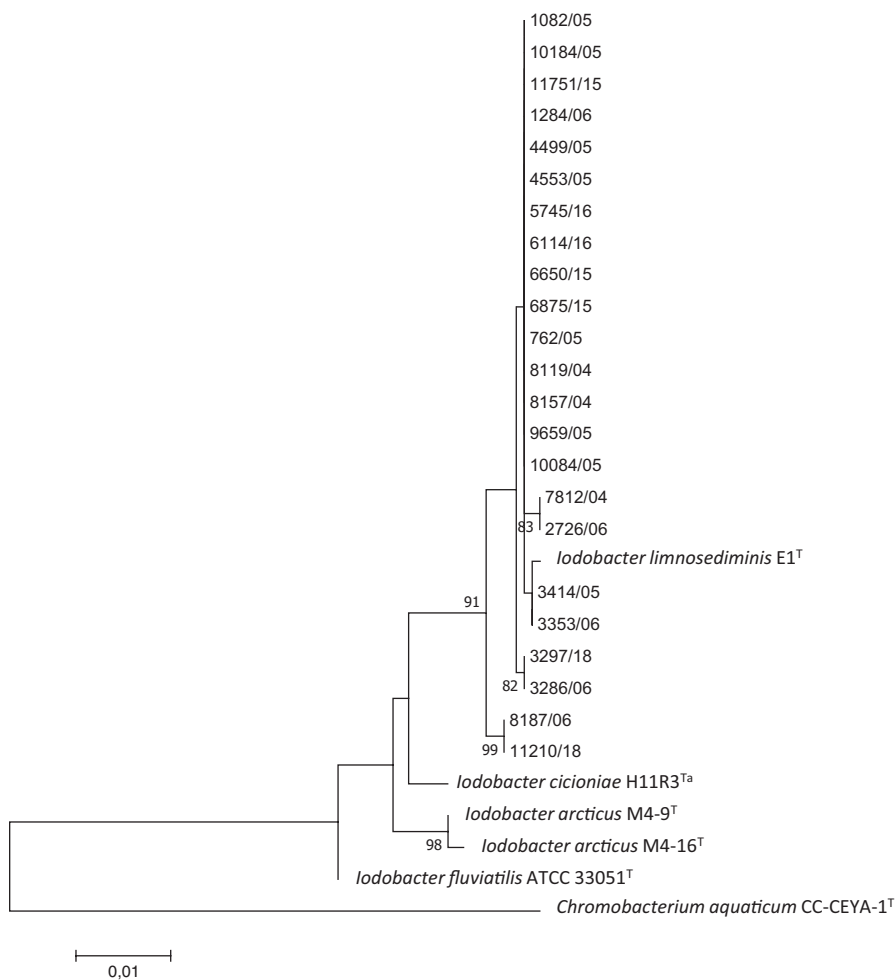


FIGURE 1 A phylogenetic tree of 16S ribosomal RNA gene of *I. limnosediminis* strains isolated from freshwater fish and related taxa constructed using the maximum-likelihood method. The tree with the highest log likelihood (-2473.39) is presented. The branch lengths in the tree are measured as the number of substitutions per site. The bootstrap value (>70) from 1,000 replicates is shown beside the branches

model with 1,000 bootstrap replicates (Figure 1). All sequence analyses were conducted using MEGA 7 software (Kumar et al., 2016).

2.4 | PFGE genotyping

The isolates were genotyped using enzyme restriction and pulsed-field gel electrophoresis (PFGE). The 16 first isolates were analysed in year 2008, and seven additional strains 6875/15, 6650/15, 11751/15, 6114/16, 5745/15, 3297/18 and 11210/18 (Table 1) were analysed in year 2020 with same methods. DNA plugs were prepared according to the PulseNet protocol (Ribot et al., 2006) with minor modifications. Briefly, bacterial cells were washed once with 1× PBS and the cell suspensions were adjusted to an OD₆₀₀ of 1.6–1.7. Proteinase K was added to the suspensions before mixing with equal volumes of 1% SeaKem Gold agarose (Lonza Walkersville Inc.) and dispensing into plug moulds. Bacterial cells were lysed overnight with proteinase K. Plugs were washed twice overnight with TEN buffer (Tris-EDTA-NaCl) at 4°C. DNA from the lysed cells was restricted with 20 units of XbaI (New England Biolabs) and loaded into 1% SeaKem Gold agarose. To separate the DNA fragments, electrophoresis was run in Bio-Rad CHEF DRIII in 0.5× TBE buffer with 6.0 V/cm and a pulse ramp from 1 to 12 s at 14°C for 16 hr. PFGE profiles were analysed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), and the results reported as a similarity dendrogram.

2.5 | Bacterial challenge experiment

To test the potential pathogenicity of *I. limnosediminis* isolated from fish skin lesions, two challenge experiments were designed: experiment 1 (exp. 1), where pathological effects for fish of two *I. limnosediminis* strains were studied, followed by experiment 2 (exp. 2) where mortality caused by passaged *I. limnosediminis* strain was assessed (Table 3). For both experiments, brown trout were acquired from a commercial hatchery and transported to the Fish Facility of the Finnish Food Authority. The fish were acclimatized in two 120-L tanks with constant water flow (average 5 L/min) of dechlorinated municipal water for 10 days. Fish were fed 1% of their body weight (Table 3) daily with commercial pellet feed (Hercules, Raisioaqua). On arrival, fish were bathed in 1:5,000 35% formaldehyde in aerated standing water for 20 min. On the third day, 10 fish per experiment were sampled randomly and health status was assessed pathologically and bacteriologically. The fish were killed with an overdose (0.2 g/L) of benzocaine (MP Biomedicals, LLC). All clinical signs were recorded, and samples were taken with a sterile loop from gills (exp. 1) and spleen (exp. 2) and streaked onto 1/4 NA, and samples from kidney were streaked on tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% magnesium sulphate heptahydrate, pH 7.2) (Whitman, 2003) (exp. 1) and 1/4 NA (exp. 2). Additionally, in exp. 2, samples were taken by aseptically scraping skin mucus from one side of the fish. The mucus was added to 450 µl

of 1× PBS in a 1.5-ml sterile tube containing 0.5 g of 0.5-mm ceramic beads (Omni International, United States) and vortexed briefly, and 100 µl was plated on 1/4 NA. The plates were incubated at 15°C for 7 days and inspected every other day for bacterial growth.

Iodobacter limnosediminis strains 8157/04 and 6875/15 isolated from the skin of brown trout with saprolegniosis (Table 1) were selected for exp. 1. *I. limnosediminis* strain 8157/04, which was reisolated from the spleen of fish injected with the same strain in exp. 1, was used as the challenge strain in exp. 2. Bacterial colonies were inoculated in TYES (exp. 1) and 1/4 NA medium (exp. 2). After 2 days of incubation at 15°C, bacterial growth was harvested by centrifugation at 4149 g for 10 min. Bacteria were resuspended in sterile TYES (exp. 1) or 1/4 NA (exp. 2) broth so that the OD₆₀₀ was 2, corresponding to approximately 1×10^9 CFU/ml. The purity and cell number of the bacterial suspensions used in the experiments were confirmed by Gram staining and 10-fold dilutions in replicate on TYES or 1/4 NA.

After 8 days of acclimation in exp. 1, feeding was stopped. On day 10, fish were randomly selected, anesthetized with benzocaine (0.05 g/L) and injected intramuscularly (i.m.) for epaxialis muscle below dorsal fin 50 µl per fish with *I. limnosediminis* 8157/04, *I. limnosediminis* 6875/15 or sterile TYES broth (control) (Table 3). After injection, the fish were kept in aerated water until they regained consciousness. The fish were then allocated between six new 120-L tanks, with 30 fish in duplicate tanks per treatment (Table 3), that is 15 fish per tank. Tank water was aerated constantly and renewed daily. Outflow water was treated with ultraviolet light (Ultraqua UV Disinfection system). The water temperature was $13.9 \pm 1.1^\circ\text{C}$ in the tanks during the 18 days of challenge in exp. 1.

At 6 days post-injection (dpi), three fish from each tank were sampled, killed with an overdose of benzocaine and inspected for disease signs. The whole spleen and a piece of the anterior kidney were aseptically removed and transferred to 450 µl of 1× PBS in a 1.5-ml sterile tube containing 0.5 g of 0.5-mm ceramic beads. Tubes with organs were vortexed for 10 s, and 100 µl of the suspension was plated on replicate 1/4 NA per sample. Observed lesions were always directly streaked onto 1/4 NA with a sterile loop. When moribund or dead fish were found during the experiment (Tables 3 and 4), they were removed, inspected and sampled accordingly. At 18 dpi, the remaining fish were sampled. The first six fish were sampled as previously described, while from the last seven fish, the spleen, kidney and skin lesions were sampled with a sterile loop and directly streaked onto 1/4 NA. The plates were incubated at 15°C for 7 days and inspected for *I. limnosediminis* colonies, and they were also screened for other pathogenic bacteria, especially for *Flavobacterium* sp. colonies, every other day. Suspected bacterial colonies, that is whitish spreading (Figure 3a) or yellow colonies, were subcultured and identified based on the colony form, Gram staining, and oxidase and catalase activities. For suspected *I. limnosediminis* colonies, further identification was performed based on individual growth tests at 37°C and biochemical tests for H-L, sucrose and mannitol assimilation, esculin hydrolysis and indole production incubated at 20°C for 2–5 days. For suspected *Flavobacterium* sp. colonies, further

identification was performed based on growth on BA and using an API ZYM Kit (BioMerieux, France) by incubating for 1–5 days at 15°C and 28°C for suspected *F. psychrophilum* and *F. columnare* colonies, respectively.

The bacterial challenge experiment was repeated in exp. 2 with *I. limnosediminis* strain 8157/04 reisolated from fish spleen in exp. 1. The experimental conditions were the same, with minor changes. Control fish were injected with 50 µl sterile 1/4 NA broth. The fish were allocated in four tanks with 34 fish per treatment in duplicate tanks (Table 3), that is 17 fish per tank. No sampling was conducted at 6 dpi in exp. 2 (Table 4). The water temperature was $14.4 \pm 1.2^\circ\text{C}$ in tanks during the 21 days of challenge exp. 2.

All the experiments were planned and conducted according to Directive 2013/63/EU on the protection of animals used for scientific purposes and the Act and Governmental Decree on the Protection of Animals Used for Scientific or Educational Purposes (497/2013 and 564/2013, respectively). Bacterial challenge experiments were reviewed and approved by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland with permission ESAVI/16637/2019.

2.5.1 | Histology

To histologically describe the impact of *I. limnosediminis* strains 8157/04 and 6875/15 on muscle tissue at the site of injection in exp. 1, samples of muscle tissue were taken from randomly selected three fish from each tank at 6 dpi. At the end of exp. 1, four fish from the *I. limnosediminis* 6875/15-challenged tanks and eight fish from the *I. limnosediminis* 8157/04-challenged tanks were sampled for histology. Muscle tissues were fixed in buffered 10% formalin and prepared according to the routine laboratory procedure. Tissue sections were stained with haematoxylin-eosin and examined by light microscopy at 50× to 100× magnification. Alterations were recorded by classifying the level of tissue degeneration and the infiltration of erythrocytes, inflammatory cells and fibrinocytes. Images of representative tissue reactions were taken using a Leica DM2500 microscope with an integrated DFC 450C camera using Leica Application Suite V4.4.0 (Leica Microsystems Ltd).

2.6 | Statistical analysis

In the bacterial challenge experiments, two tank variables with the same treatment were compared with the chi-square test (all comparisons non-significant, $p > .05$) before the tanks were combined as one treatment for further analysis. The statistical significance of lesion appearance and *I. limnosediminis* isolation was compared between the *I. limnosediminis* challenge treatment and control treatment with Fisher's exact tests. The difference in survival between control and *I. limnosediminis*-injected fish in exp. 2 was analysed with the Kaplan–Meier log-rank test. All the statistical analyses were performed with IBM SPSS Statistics 25.0 Software.

3 | RESULTS

3.1 | Clinical signs and bacteriology of the sampled fish

Pathological findings from diseased fish were consistently skin lesions (Table 1). For four studied freshwater bream, the lesions comprised one or more ulcers on the flanks. For salmonid fish, one rainbow trout (*Oncorhynchus mykiss*) developed skin lesions without saprolegniosis and the rest of the salmonid fish; that is, two rainbow trout, 11 brown trout, three Atlantic salmon (*Salmo salar*) and two whitefish (*Coregonus lavaretus*), had lesions dominated by saprolegniosis. No other pathological findings, beside the skin lesions, were usually recorded. However, abnormal spleen enlargement and/or fin rot were observed for three salmonid fish. The isolations were made from variable sizes of fish. Primary cultures from the skin and/or lesions of fish were mostly mixed cultures including colonies of *I. limnosediminis*. Most of the *I. limnosediminis* colonies were isolated from 1/4 NA (21/23) and two from AOA medium. Atypical *Aeromonas salmonicida* and *A. hydrophila* were also found in one primary culture from freshwater bream, and *Flavobacterium psychrophilum* was additionally identified from five primary cultures from salmonid fish.

3.2 | Phenotypic and biochemical characteristics

All *I. limnosediminis* strains isolated from fish formed whitish colonies with spreading growth on 1/4 NA (Figure 3a). Every studied strain was rod-shaped, Gram negative and catalase, oxidase and nitrate reduction positive (Table 2). When glucose assimilation and fermentation was tested with H-L at 20°C, all *I. limnosediminis* strains were positive, including *I. limnosediminis* DSM 103822^T. Motility in H-L tubes was also observed. After incubation for 2 days at 20°C, sucrose assimilation was also positive and mannitol assimilation, esculin hydrolysis and indole production were consistently negative in all strains tested.

Contrary to the H-L test, in API 20 NE after incubation at 28°C, glucose fermentation and assimilation were usually negative for the *I. limnosediminis* strains isolated from fish and for *I. limnosediminis* DSM 103822^T and *I. fluviatilis* DSM3764^T (Table 2). Type strain *I. limnosediminis* DSM 103822^T was positive or weakly positive for the assimilation of arabinose, N-acetylglucosamine, maltose and gluconate, while variable results were obtained for *I. limnosediminis* strains isolated from fish with API 20 NE (Table 2). The assimilation of mannitol, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid was negative for all the strains studied. The most common API 20 NE profile for fish isolates of *I. limnosediminis* (5/24) was 1043704 in APIWEB™, with 88.3% identity with *Ochrobactrum anthropi*. The strain *I. limnosediminis* DSM 103822^T and two other strains (3/24) had APIWEB™ profile 1003704, with 97.1% identity with *O. anthropi*.

In MALDI-TOF analysis, the identification spectra were set with scores between 0 and 3, where a logarithmic score cut-off of ≥ 2 indicates high-confidence identification (MBT Compass User Manual

TABLE 2 Comparison of biochemical and physiological characteristics between *Iodobacter limnosediminis* isolated from fish and type strains *I. limnosediminis* DSM 103822^T and *I. fluviatilis* DSM 3764^T

Characteristics	<i>I. limnosediminis</i> strains isolated from fish (n = 23)	<i>I. limnosediminis</i> DSM 103822 ^T	<i>I. fluviatilis</i> DSM 3764 ^T
Gram staining	-	-	-
Spreading growth	+	+	+
Violet pigmentation	-	-	+
Growth at 37°C	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Incubation at +28°C			
Nitrate reduction	+	+	+
Indole production	-	-	-
Glucose fermentation	22 ^a	-	-
Arginine dihydrolase	-	-	-
Urease	-	-	-
β-Glucosidase	-	-	-
Gelatin hydrolysis	-	-	-
β-Galactosidase	-	-	-
Glucose assimilation	65 ^a	-	+
Arabinose assimilation	65 ^a	(+)	-
Mannose assimilation	96 ^a	+	(+)
Mannitol assimilation	-	-	-
N-acetylglucosamine assimilation	91	(+)	+
Maltose assimilation	67 ^a	+	+
Gluconate assimilation	91 ^a	(+)	+
Incubation at +20°C			
Glucose fermentation	+	+	+
Glucose assimilation	+	+	+
β-Glucosidase	-	-	-
Mannitol assimilation	-	-	-
Sucrose assimilation	+	+	-
Indole	-	-	-

Note: Biochemical tests were conducted with an API 20 NE system at +28°C and with individual tests at +20°C. +, 100% positive; -, 100% negative; (+), weakly positive; numbers represent the percentage of positive reactions.

^aWeakly positive, included as positive.

Revision E, April 2019, Bruker). All 24 of the tested *I. limnosediminis* strains had a score of over 2 when compared to the *I. limnosediminis* DSM 103822^T MSP, while *I. fluviatilis* DSM 3764^T had a score of <1.7. The strain *I. fluviatilis* DSM 3764^T scored over 2 for *I. fluviatilis* MSP (Bruker database). However, *I. fluviatilis* DSM 3764^T was occasionally identified as *I. limnosediminis* with a score between 1.95 and 1.77, that is with low confidence in the identification.

3.3 | 16S rRNA gene sequencing

The 16S rRNA gene sequences were identical for 15 *I. limnosediminis* strains isolated from fish (Figure 1). These strains had only

two nucleotide differences compared to *I. limnosediminis* type strain E1. One of these 15 identical sequences, that is *I. limnosediminis* 8157/04, was compared with sequences of bacterial strains with a BLAST search, which indicated 99.5% identity with *I. limnosediminis* E1^T; 98.3% identity with *I. ciconiae* H11R3^T; 98.1% and 97.9% identity with *I. arcticus* strains M4-9^T and M4-16^T, respectively; and 95.6% identity with *I. fluviatilis* strain ATCC 33051^T. Interestingly, 99.9% identity was found with a partial 16S rRNA gene sequence of *Iodobacter* sp. LE23 (accession no. FN908444), isolated from a lesion with saprolegniosis on brown trout in Spain and submitted by the authors, Carbajal-González et al. (2011).

All the 16S rRNA gene sequences of *I. limnosediminis* strains isolated from fish grouped in the same branch of the phylogenetic tree,

forming a distinctive clade separate from the other *Iodobacter* spp with a high bootstrap value (91%) (Figure 1). In phylogenetic tree, two *I. limnosediminis* strains 11210/18 and 8187/06 formed a separate clade and had nine nucleotide variabilities in their 16S rRNA gene compared to type strain *I. limnosediminis* E1^T. These strains were both isolated from farmed fish with saprolegniosis from the Kymijoki watercourse (Figure 1 and Table 1).

3.4 | PFGE

There was approximately 60% similarity between 23 *I. limnosediminis* strains studied using whole-genome restriction analysis with PFGE. Two strains, 762/05 and 10084/05, were identical in the analysis, even though they originated from different water courses and different fish hosts. However, beside these identical strains, all the other strains had high variation between the restriction profiles, and no host, spatial or temporal clustering was observed between them (Figure 2).

3.5 | Bacterial challenge experiments

In experiments 1 and 2, no pathological disease signs, bacterial growth in internal organs or pathogenic bacteria or saprolegniosis on the skin or gills were detected from fish before the experiments

($n = 10$ per experiment). In exp. 1 at 6 dpi, severe changes were recorded at the site of injection in most of the fish injected with *I. limnosediminis*. At 6 dpi, 50% (3/6) of sampled fish from the *I. limnosediminis* 6875/15 challenge treatment and 83% (5/6) of sampled fish from the 8157/04 challenge treatment had lesions at the site of injection. These fish had a visibly damaged area of skin and muscular tissue often involving further areas around the site of injection (Figure 3b). No lesions were detected in the control fish ($n = 6$). At 6 dpi, *I. limnosediminis* was re-isolated from half of the sampled fish in challenge treatments from lesions, spleen and/or kidney, but not from the control fish (Table 4). Between 8 and 13 dpi, three dead/moribund fish from among the *I. limnosediminis*-injected fish were sampled (Table 4). The dead fish also displayed disease signs such as fin rot and pale gills and skin, and the site of injection had developed into an open ulcer (Figure 3c). One dead fish had splenomegaly. No mortality was recorded in the control groups (Table 3). In exp. 1, *I. limnosediminis* was isolated from five fish from both challenge treatment groups but none of the control fish (Table 4). There were significantly more *I. limnosediminis* isolations from the 8157/04 challenge treatment (Fisher's exact test, $p = .013$) and 6875/15 challenge treatment (Fisher's exact test, $p = .026$) than from the control treatment. The difference in the number of lesions recorded in fish challenged with *I. limnosediminis* 6875/15 and 8157/04 compared to the control group was statistically significant (Table 3). At 18 dpi, at the end of exp.1, the sampled fish had lesions that appeared to be healing, with scar tissue at the site of injection.

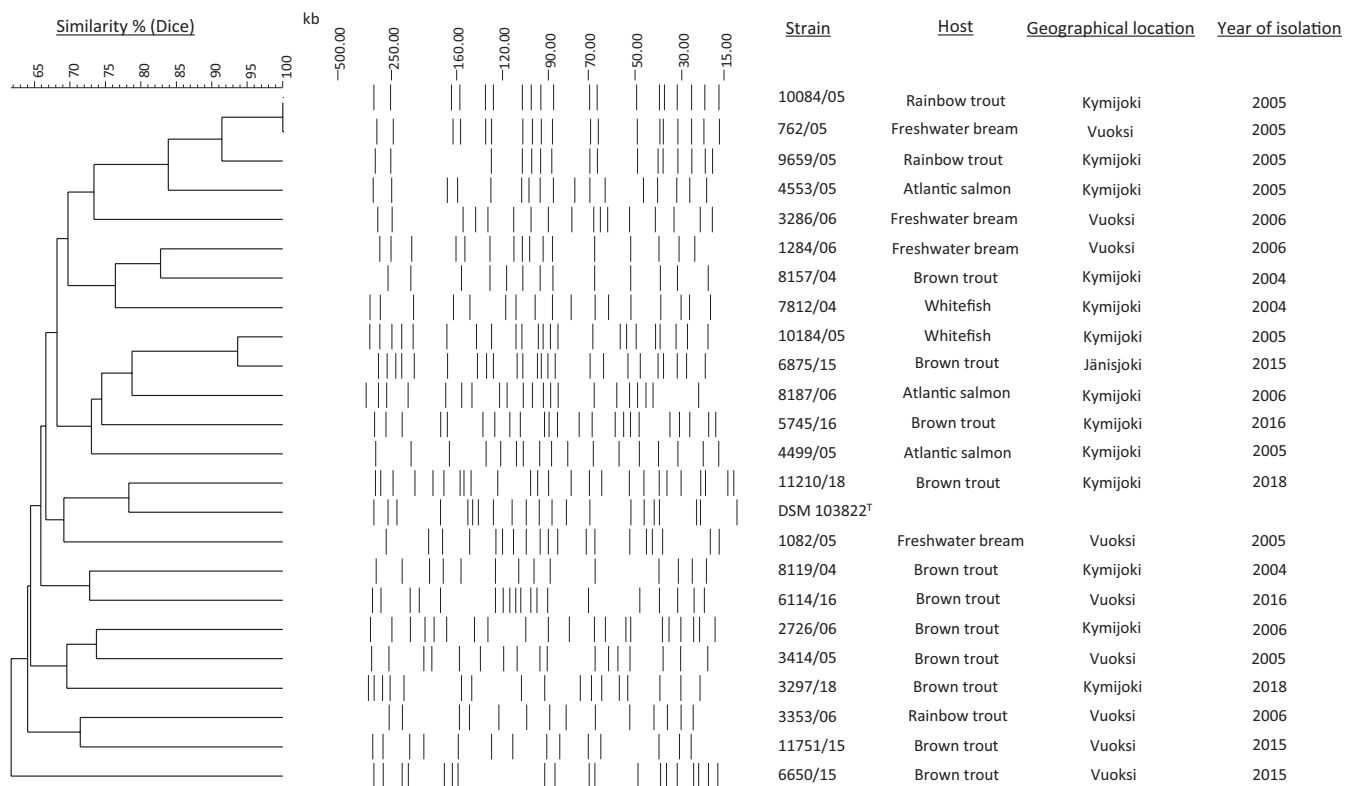


FIGURE 2 A similarity dendrogram of whole-genome PFGE banding patterns of the *I. limnosediminis* isolates using the unweighted pair-group method with arithmetic mean (UPGMA) in BioNumerics software. The strain, host, geographical location as the watercourse in Finland and year of isolation are presented

In exp. 2, the cumulative mortality of passaged 8157/04 *I. limnosediminis*-challenged fish was 6% compared to 0% among control fish. There was no significant difference in survival between the groups (log-rank test, $p = .154$). Two dead/moribund fish had an open ulcer at the site of injection and pale skin, and additionally, the other fish had fin rot. The percentage of lesions recorded from all the fish by the end of the experiment was statistically higher among the challenged fish than the control fish (Table 3). *I. limnosediminis* was isolated from the lesions of two dead fish, but no bacteria were isolated from any fish sampled at the end of experiment 2 at 21 dpi (Table 4), and the number of isolations was not significantly higher in the challenge treatment compared to the control treatment (Fisher's exact test, $p = .246$) in exp. 2.

3.5.1 | Histology

At 6 dpi, some of the control fish (3/6) developed minor tissue damage at the site of injection, where minor accumulations of erythrocytes and lymphocytes and disruption of individual myotomes were detected (Figure 4a). On the contrary, in the groups injected with *I. limnosediminis* strains 6875/15 and 8157/04, severe muscular damage was detected in 9 of the 12 fish sampled for histopathology at 6 dpi. Massive accumulation of leucocytes was observed, spreading into the muscle tissue between the myotomes and infiltrating the necrotic myotomes (Figure 4b). Bacterial masses were still seen intramuscularly in four of the fish. By the end of exp. 1, at 18 dpi, the main finding was the development from an acute infective reaction to repair of the tissue by fibrocytes and macrophages (Figure 4c) seen in 11 out of 12 fish sampled for histology from the challenge treatments. However, two of these fish had developed an open ulcer that had not healed by the time of sampling and left the underlying tissue unprotected by skin (Figure 4d).

4 | DISCUSSION

There have been no previous reports on the characterization of *I. limnosediminis* isolated from freshwater fish. *I. limnosediminis* was first described in 2013 (Su et al., 2013), and to our knowledge, there have been no publications on the species since then. This bacterial species could have been overlooked, although it might be relatively common in temperate freshwater aquatic systems and on freshwater fish surfaces according to the present study and that of Carbajal-Gonzalez et al. (2011). We isolated *I. limnosediminis* from several freshwater fish and characterized it phenotypically and genetically to help the future recognition of this bacterial species. Sequence analysis of the 16S rRNA gene revealed that the studied strains all belonged genetically to the same species, and they were distinguished from other closely related *Iodobacter* spp. The whole-genome restriction fragment analysis with PFGE supported the argument that all the *I. limnosediminis* strains isolated were same species, although there was more variability between the strains in PFGE than in 16S rRNA gene

sequencing, and no patterns were detected in the host, geographical area or year of isolation. This suggests that there are multiple environmental sources for *I. limnosediminis* isolated from fish. Two *I. limnosediminis* strains were genetically identical according to the PFGE and 16S rRNA gene sequence analysis and were isolated in the same year but from different host species and geographical areas. However, epidemiological links may exist between these areas in the form of stockings of fish for angling purposes.

Regarding phenotypic and biochemical characteristics, the *I. limnosediminis* strains collected from fish were very similar to the

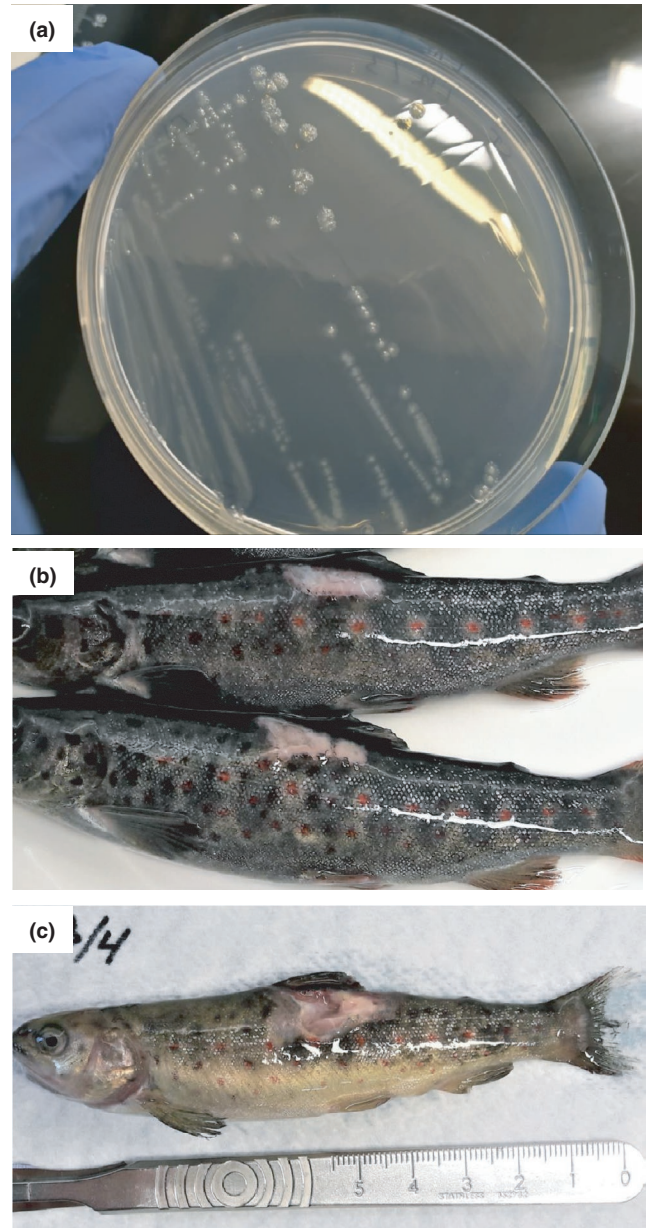


FIGURE 3 Whitish spreading colonies of *I. limnosediminis* on 1/4 NA at 15°C after 6 days of incubation (a). Lesions at the site of injection close by the dorsal fin on brown trout sampled at 6 dpi after injection with *I. limnosediminis* strain 8157/04 (b). An open ulcer involving both muscular tissue and skin, as well as paleness and fin rot, in a fish that died at 11 dpi (c). Dpi, days post-injection

TABLE 3 Information on the parameters in bacterial challenges with *Iodobacter limnosediminis* 8157/04 and *I. limnosediminis* 6,875 in experiment 1 and passaged *I. limnosediminis* 8157/04 in experiment 2

Experiment	Challenge treatment	No. of fish	Average weight (g ± SD)	Dose (CFU/fish)	Percentage of fish with lesions	No. of dead/moribund fish terminated
1	<i>I. limnosediminis</i> 8157/04	30	16.6 ± 0.5	5 × 10 ⁷	53% ^a	2
	<i>I. limnosediminis</i> 6875/15	30	16.6 ± 0.5	4 × 10 ⁷	27% ^a	1
	Control	30	16.0 ± 0.5	n/a	0%	0
2	Passaged <i>I. limnosediminis</i> 8157/04	34	13.5 ± 2.3	5 × 10 ⁶	41% ^a	2
	Control	34	12.9 ± 2.2	n/a	0%	0

Note: The percentages of fish with lesions at the end of experiments.

^aSignificantly different from the control group (Fisher's exact test, $p \leq .005$)

type strain *I. limnosediminis* DSM 103822^T. Obviously, this relatively unknown bacterial species has no API-based species recognition. However, the most common API 20 NE profiles in our results for *I. limnosediminis* were 1043704 and 1003704, also including the strain *I. limnosediminis* DSM 103822^T, and the API results were relatively consistent with API 20 NE results reported by Su et al. (2013). However, several biochemical tests yielded variable results in API 20 NE when performed according to the manufacturer's instructions and after incubation at 28°C. We noticed that more consistent results from biochemical analysis and identification of the *I. limnosediminis* strains were obtained in individual tests at 20°C. For example, both glucose assimilation and fermentation were positive for all the *I. limnosediminis* strains, including the *I. limnosediminis* DSM 103822^T, but when analysed with API 20NE, only 66% and 22% were positive, respectively, and the type strain was negative for both glucose assimilation and fermentation (Table 2). To avoid inaccurate identification of *I. limnosediminis*, we applied a few phenotypic and biochemical tests at 20°C that were consistent; that is, *I. limnosediminis* strains did not produce violet pigment and were sucrose positive, unlike *I. fluviatilis* and *I. arcticus* (Logan, 1989; Srinivas et al., 2013), and were unable to grow at 37°C, contrary to motile *Aeromonas* spp. On 1/4 NA, the typical spreading growth of the genus *Iodobacter* can be observed, making the differentiation between the aeromonads and *Iodobacter* sp. clearer, while these bacterial species are easily confused when examining the colony form on common media such as BA. Indole production, esculin hydrolysis and mannitol assimilation were also always negative in the studied *I. limnosediminis* strains at 20°C, while they are usually positive for several motile *Aeromonas* sp. isolated from diseased fish (Beaz-Hidalgo et al., 2010; Whitman, 2003). In *A. salmonicida*, hydrolysis of esculin is in most cases positive, except for *A. salmonicida* subsp. *achromogenes* and subsp. *smithia* (Whitman, 2003). However, being non-motile and also differing in colony form, these species are easily recognizable. Esculin is also positive for another non-violet-pigmented *Iodobacter* sp, *I. ciconiae* (Lee et al., 2019). MALDI-TOF, a technique that is based on mass spectrometry of protein fingerprinting, is considered to be a rapid and accurate identification method for several pathogenic fish bacteria (Jansson et al., 2020). Phenotypically, all the *I. limnosediminis* strains isolated from fish lesions were identified with MALDI-TOF analysis with high confidence. Furthermore, the

I. limnosediminis strains could be identified by MALDI-TOF in agreement with morphology and growth characteristics, indicating a high confidence identification to species level (Jansson et al., 2020).

A low virulent strain of the opportunistic fish pathogen *F. columnare* displayed higher virulence in a high nutrient environment rather than with higher bacterial doses in a fish challenge model (Kinnula et al., 2017). Indeed, environmental opportunistic bacteria are known to have the ability to change virulence in different environmental circumstances, as they often live both in and outside the host (Brown et al., 2012). According to the findings of this study, along with genetic analysis, *I. limnosediminis* is most likely an environmental bacterium, the clinical significance of which remains unclear, as it was rarely isolated as a monoculture. However, several bacterial species have been shown to cause skin lesions in fish and often coexist on fish surfaces (Austin & Austin, 2016). Some of these, *Aeromonas* spp. and *F. psychrophilum*, were also found coexisting with *I. limnosediminis* in lesions on five diseased fish from which *I. limnosediminis* was isolated in this study. However, for most of the fish (18/23) from which *I. limnosediminis* was originally isolated, no known pathogenic bacteria were detected in primary cultures. The 1/4 NA used in the primary isolation process allows the typical spreading growth of the genus *Iodobacter*, while at the same time suppressing the growth of many ubiquitous bacterial species. Although many different low nutrient media have been used for the detection of flavobacteria (Song et al., 1988, Austin & Austin, 2016), 1/4 NA has been used for the detection of aquatic species (Moss et al., 1978) and as such is not widely used in fish disease diagnostics. However, *F. columnare* and *F. psychrophilum* also grow on this same medium, even allowing simultaneous detection of these two genera when present (own unpublished results).

In the challenge experiments, causality between *I. limnosediminis* isolated from diseased fish and disease signs was suggested; that is, skin lesions were observed when *I. limnosediminis* was introduced to healthy fish and the bacterium was reisolated from fish with lesions and identified as *I. limnosediminis*. Both the appearance of lesions in experiments 1 and 2 and *I. limnosediminis* isolation in experiment 1 were significantly more frequent in the challenge treatments than in the control treatments. This suggests that *I. limnosediminis* could be capable of causing lesions in fish under certain circumstances. However, mortality was not significant, the bacteria were rarely

TABLE 4 *Iodobacter limnosediminis* isolations from fish and tissues sampled during experiments 1 and 2

Experiment	Challenge treatment	Sample 6 dpi			Mortality/moribund fish terminated 8–13 dpi						Sample 18 dpi (exp. 1) and 21 dpi (exp. 2)			
		Isolations/no. of fish			Lesion	Spleen	Kidney	Isolations / no. of fish			Lesion	Spleen	Kidney	
		Lesion	Spleen	Kidney				Lesion	Spleen	Kidney				
1	<i>I. limnosediminis</i> 8157/04	3/6	0	3	1	1	1/2	1	1	0	1/22	0	1	0
	<i>I. limnosediminis</i> 6875/15	3/6	3	1	1	1/1	1	1	0	0	1/23	1	0	0
	Control	0/6	0	0	0	0	0	0	0	0	0/24	0	0	0
2	Passaged <i>I. limnosediminis</i> 8157/04	n/a	n/a	n/a	n/a	2/2	2	0	0	0	0/32	0	0	0
	Control	n/a	n/a	n/a	n/a	0	0	0	0	0	0/34	0	0	0

Abbreviation: Dpi, days post-injection.

recovered from inner organs, the lesions in the fish had often healed by the end of the experiments, and *I. limnosediminis* was not isolated from the fish at the end of experiments. This reflects the clinical findings from the original fish hosts, as no systemic infections were recorded as being caused by this bacterial species.

The injection challenge model used in this study bypasses external defence mechanisms of fish. However, it has earlier been recognized that bacterial pathogens may not give reproducible results in waterborne challenge models (Decostere et al., 2000), especially with low virulent bacteria. Partly for this reason laboratory infections are often done by injection (Fredriksen et al., 2013; Sundell et al., 2019), as was also done in this study. Indeed, histopathological examination revealed severe local destruction of tissues in the experimental infection, but in most cases the damage was restricted, and the tissue subsequently repaired via the accumulation of fibrocytes. However, especially in dead and moribund fish that were terminated during the challenge experiments, the damage was severe enough to leave an open ulcer. Several fish pathogens can cause skin lesions in fish (Austin & Austin, 2016), which can advance to open ulcers. Similar muscular ulceration as described in this study has been reported for example from fish pathogens *Chryseobacterium* sp. and *Flavobacterium* sp. (Bruce et al., 2020). Further studies are needed to determine environmental circumstances, the route of invasion and proteolytic/cytotoxic activity of *I. limnosediminis*, to study whether it has similar affect on fish as for example *F. psychrophilum* (Iturriaga et al., 2017; Ostland et al., 2000).

Over the last 20 years, *I. limnosediminis* has been repeatedly isolated from diseased fish in Finland (data not shown), and almost all *I. limnosediminis* strains investigated here were isolated from salmonids with saprolegniosis (18 of 19 salmonids sampled had saprolegniosis). In an earlier report from Spain, *Iodobacter* sp. was most frequently isolated from salmonid fish with saprolegniosis along with *Aeromonas* sp. (Carbajal-Gonzalez et al., 2011). In the same study, *Iodobacter* sp. and *Aeromonas* sp. were also found on healthy brown trout skin, but not on healthy rainbow trout skin. Saprolegniosis is a common disease, especially in salmonids, and is caused by aquatic oomycetes of *Saprolegnia* spp. Most *Saprolegnia* spp. are considered parasitic or opportunistic pathogens in fish, usually attacking damaged skin and immunocompromised and/or injured individuals. The fish pathogen *S. parasitica* is assumed to be adapted to parasitism on fish by producing spores with hooks and adhesive compounds, which facilitate attachment to the host (Dieguez-Urbeondo et al., 2007; Rezinciuc et al., 2018). When attached, *Saprolegnia* sp. forms lesions on the fish surface and might cause progressive dermatomycosis by penetrating the epidermis with hyphae. Saprolegniosis has been reported to coexist with other pathogens, and lesions on fish surfaces accumulate greater numbers of bacteria than healthy fish skin (Austin & Austin, 2016). Interestingly, some *Aeromonas* spp. have an inhibitory effect on *S. parasitica*, and it has been suggested that one of the reasons why some fish recover from saprolegniosis is partly because of inhibitory bacteria in the skin microflora (Carbajal-Gonzalez et al., 2011; Lategan & Gibson, 2003). No inhibitory effect of *Iodobacter* sp. against *S. parasitica* was found in

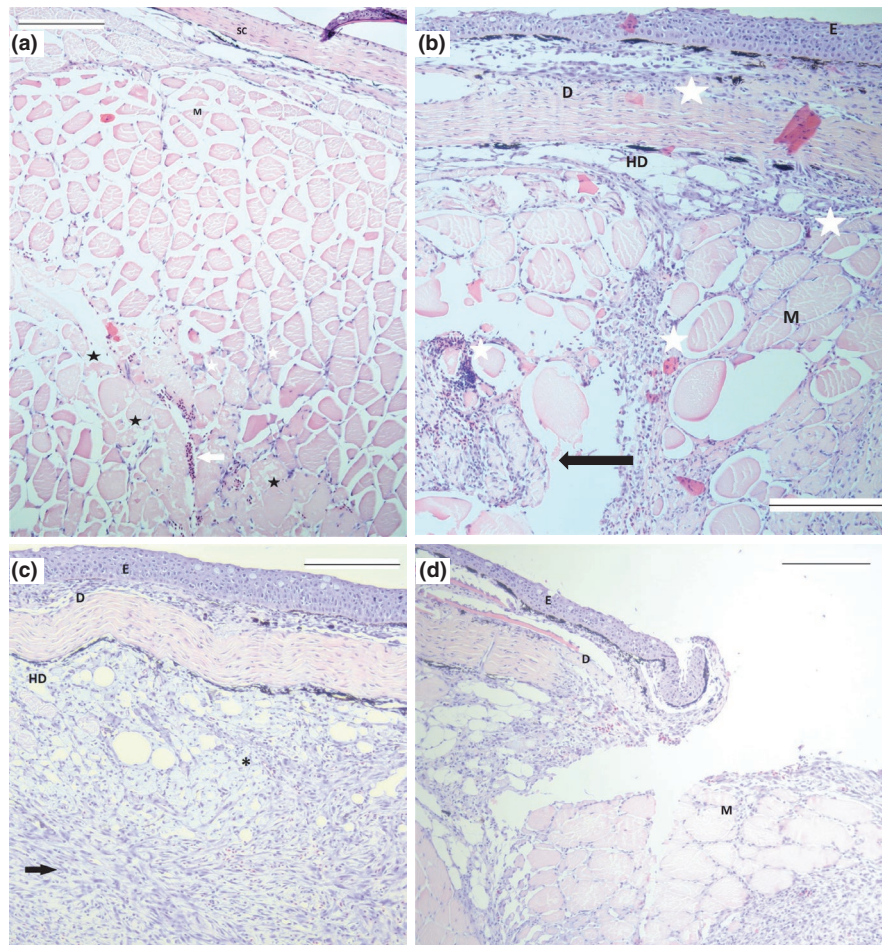


FIGURE 4 Histopathological changes in brown trout from experiment 1. Muscle lesion in a control fish injected with sterile broth at 6 dpi. Minor degeneration of muscle cells (black stars) and accumulation of leucocytes (white stars), as well as some erythrocyte (white arrow), can be seen at the injection site (a). Muscle lesion at 6 dpi in a fish injected with *I. limnosediminis* strain 8157/04. Massive influx of leucocytes (white stars) has occurred into the muscle, as well as the dermis and hypodermis, and the myotomes are focally degenerated (black arrow) and invaded by leucocytes (b). Muscle lesion at 18 dpi in a fish injected with *I. limnosediminis* strain 8157/04. Muscle tissue and hypodermal tissue have been replaced mainly by fibrocytes (black arrow) and macrophages (asterisk) (c). Muscle lesion at 18 dpi in a fish injected with *I. limnosediminis* strain 8157/04. The muscle tissue has not recovered and is exposed, lacking layers of epidermis and dermis (d). M: muscle tissue E: epidermis D: dermis HD: hypodermis SC: stratum compactum. Dpi: days post-injection. H&E, scale bars 200 μ m

vitro (Carbajal-Gonzalez et al., 2011). Indeed, concurrent infections might have suppressive, synergistic or additive effects on fish health (Cox, 2001). Parasitic infections are often described as being more severe for fish health when occurring in connection with bacterial pathogens (Carvalho et al., 2020; Cox, 2001; Figueroa et al., 2017). Further research is needed to determine whether bacteria, especially those causing skin lesions, can facilitate and/or enhance saprolegniosis on fish surfaces.

Although challenge with *I. limnosediminis* did not induce significant mortality in brown trout in this study, the injection led to severe tissue damage in several fish. This study revealed a possible interaction between *I. limnosediminis* and fish in temperate freshwater aquatic environments. This relatively unknown bacterial species was frequently recovered from diseased fish, and its role in the pathogenesis of skin disorders warrants further study involving several fish species and environmental conditions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

16S rRNA sequence data (accession no. MW553175–MW553197) are available in the GenBank database (<https://www.ncbi.nlm.nih.gov>). All the other data are available in this manuscript and on request from the authors.

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REFERENCES

- Angen, Ø., Ahrens, P., & Tegtmeier, C. (1998). Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. *Veterinary Microbiology*, 63(1), 39–48. [https://doi.org/10.1016/S0378-1135\(98\)00222-3](https://doi.org/10.1016/S0378-1135(98)00222-3)
- Austin, B. (2006). The Bacterial Microflora of Fish, Revised. *The Scientific World Journal*, 6, 325830. <https://doi.org/10.1100/tsw.2006.181>
- Austin, B., & Austin, D. (2016). *Bacterial Fish Pathogens, Disease of Farmed and Wild Fish*. Springer International Publishing.
- Beaz-Hidalgo, R., Alperi, A., Buján, N., Romalde, J. L., & Figueras, M. J. (2010). Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. *Systematic and Applied Microbiology*, 33(3), 149–153. <https://doi.org/10.1016/j.syapm.2010.02.002>
- Bernardet, J. F., & Kerouault, B. (1989). Phenotypic and genomic studies of "Cytophaga psychrophila" isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Applied and Environmental Microbiology*, 55(7), 1796–1800. <https://doi.org/10.1128/aem.55.7.1796-1800.1989>
- Brown, S., Cornforth, D., & Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: Generalism, plasticity, and control. *Trends in Microbiology*, 20(7), 336–342. <https://doi.org/10.1016/j.tim.2012.04.005>
- Bruce, T. J., Ma, J., Knupp, C., Loch, T. P., Faisal, M., & Cain, K. D. (2020). Cross-protection of a live-attenuated *Flavobacterium psychrophilum* immersion vaccine against novel *Flavobacterium* spp. and *Chryseobacterium* spp. strains. *Journal of Fish Diseases*, 43(8), 915–928. <https://doi.org/10.1111/jfd.13201>
- Carbajal-González, M. T., Fregeneda-Grandes, J. M., Suárez-Ramos, S., Rodríguez Cadenas, F., & Aller-Gancedo, J. M. (2011). Bacterial skin flora variation and in vitro inhibitory activity against *Saprolegnia parasitica* in brown and rainbow trout. *Diseases of Aquatic Organisms*, 96(2), 125–135. <https://doi.org/10.3354/dao02391>
- Carvalho, L. A., Whyte, S. K., Braden, L. M., Purcell, S. L., Manning, A. J., Muckle, A., & Fast, M. D. (2020). Impact of co-infection with *Lepeophtheirus salmonis* and *Moritella viscosa* on inflammatory and immune responses of Atlantic salmon (*Salmo salar*). *Journal of Fish Diseases*, 43(4), 459–473. <https://doi.org/10.1111/jfd.13144>
- Cox, F. E. G. (2001). Concomitant infections, parasites and immune responses. *Parasitology*, 122(S1), S23–S38. <https://doi.org/10.1017/S003118200001698X>
- Decostere, A., Lammens, M., & Haesebrouck, F. (2000). Difficulties in experimental infection studies with *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) using immersion, oral and anal challenges. *Research in Veterinary Science*, 69, 165–169. <https://doi.org/10.1053/rvsc.2000.0408>
- Diéguez-Urbeondo, J., Fregeneda-Grandes, J. M., Cerenius, L., Pérez-Iniesta, E., Aller-Gancedo, J. M., Tellería, M. T., Söderhäll, K., & Martín, M. P. (2007). Re-evaluation of the enigmatic species complex *Saprolegnia diclina*-*Saprolegnia parasitica* based on morphological, physiological and molecular data. *Fungal Genetics and Biology*, 44(7), 585–601. <https://doi.org/10.1016/j.fgb.2007.02.010>
- Figuerola, C., Bustos, P., Torrealba, D., Dixon, B., Soto, C., Conejeros, P., & Gallardo, J. (2017). Coinfection takes its toll: Sea lice override the protective effects of vaccination against a bacterial pathogen in Atlantic salmon. *Scientific Reports*, 7(1), 17817. <https://doi.org/10.1038/s41598-017-18180-6>
- Fredriksen, B. N., Furevik, A., Gauthier, D., Egenberg, M., Paulsen, E. D., & Brudeseth, B. (2013). Intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*) with two Norwegian field strains of *Flavobacterium psychrophilum*. *Fish & Shellfish Immunology*, 35, 595–598. <https://doi.org/10.1016/j.fsi.2013.04.037>
- Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., & Bailey, M. J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*, 66(12), 5488–5491. <https://doi.org/10.1128/aem.66.12.5488-5491.2000>
- Iturriaga, M., Espinoza, M. B., Poblete-Morales, M., Feijoo, C. G., Reyes, A. E., Molina, A., Avendaño-Herrera, R., & Valdés, J. A. (2017). Cytotoxic activity of *Flavobacterium psychrophilum* in skeletal muscle cells of rainbow trout (*Oncorhynchus mykiss*). *Veterinary Microbiology*, 210, 101–106. <https://doi.org/10.1016/j.vetmic.2017.09.009>
- Jansson, E., Haenen, O., Nonnemann, B., Madsen, L., van Gelderen, E., Aspán, A., Saker, E., Boonstra, M., Gulla, S., Colquhoun, D. J., Roozenburg-Hengst, I., & Dalsgaard, I. (2020). MALDI-TOF MS: A diagnostic tool for identification of bacterial fish pathogens. *Bulletin of the European Association of Fish Pathologists*, 40(6), 240–248. https://eafp.org/download/2020-volume40/issue_6/40-6-240-jansson.pdf
- Kinnula, H., Mappes, J., Valkonen, J. K., Pulkkinen, K., & Sundberg, L.-R. (2017). Higher resource level promotes virulence in an environmentally transmitted bacterial fish pathogen. *Evolutionary Applications*, 10(5), 462–470. <https://doi.org/10.1111/eva.12466>
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Lane, D. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt, & M. Goodfellow (Eds.), *Nucleic acid techniques in bacterial systematics* (pp. 115–175). John Wiley & Sons.
- Lategan, M. J., & Gibson, L. F. (2003). Antagonistic activity of *Aeromonas media* strain A199 against *Saprolegnia* sp., an opportunistic pathogen of the eel, *Anguilla australis* Richardson. *Journal of Fish Diseases*, 26, 147–153. <https://doi.org/10.1046/j.1365-2761.2003.00443.x>
- Lee, J.-Y., Kang, W., Kim, P. S., Lee, S.-Y., Shin, N.-R., Sung, H., Lee, J.-Y., Yun, J.-H., Jeong, Y.-S., Han, J. E., Jung, M.-J., Hyun, D.-W., Kim, H. S., Tak, E. J., Kang, M.-S., Lee, K.-E., Lee, B.-H., & Bae, J.-W. (2019). *Iodobacter ciconiae* sp. nov., a bacterium isolated from feces of oriental stork, *Ciconia boyciana*. *International Journal of Systematic and Evolutionary Microbiology*, 69(9), 2948–2953. <https://doi.org/10.1099/ijsem.0.003583>
- Loch, T. P., & Faisal, M. (2015). Emerging flavobacterial infections in fish: A review. *Journal of Advanced Research*, 6(3), 283–300. <https://doi.org/10.1016/j.jare.2014.10.009>
- Logan, N. (1989). Numerical taxonomy of violet-pigmented, gram-negative bacteria and description of *Iodobacter fluviatile* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 39(4), 450–456. <https://doi.org/10.1099/00207173-39-4-450>
- Mac Faddin, J. F. (1980). *Biochemical tests for identification of medical bacteria*. The Williams & Wilkins company.
- Moss, M. O., Ryall, C., & Logan, N. A. (1978). The classification and characterization of chromobacteria from a lowland river. *Microbiology*, 105(1), 11–21. <https://doi.org/10.1099/00221287-105-1-11>
- Ostland, V. E., Byrne, P. J., Hoover, G., & Ferguson, H. W. (2000). Necrotic myositis of rainbow trout, *Oncorhynchus mykiss* (Walbaum): Proteolytic characteristics of a crude extracellular preparation from *Flavobacterium psychrophilum*. *Journal of Fish Diseases*, 23(5), 329–336. <https://doi.org/10.1046/j.1365-2761.2000.00251.x>
- Pękala, A., Kozińska, A., Paździor, E., & Głowacka, H. (2015). Phenotypical and genotypical characterization of *Shewanella putrefaciens* strains isolated from diseased freshwater fish. *Journal of Fish Diseases*, 38(3), 283–293. <https://doi.org/10.1111/jfd.12231>
- Pękala, A., Paździor, E., Antychowicz, J., Bernad, A., Głowacka, H., Więcek, B., & Niemczuk, W. (2018). *Kocuria rhizophila* and *Micrococcus luteus* as emerging opportunist pathogens in brown trout (*Salmo trutta*

- Linnaeus, 1758) and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). *Aquaculture*, 486, 285–289. <https://doi.org/10.1016/j.aquaculture.2017.12.028>
- Pełkala-Safińska, A. (2018). Contemporary threats of bacterial infections in freshwater fish. *Journal of Veterinary Research*, 62(3), 261–267. <https://doi.org/10.2478/jvetres-2018-0037>
- Rezinciuc, S., Sandoval-Sierra, J. V., Ruiz-León, Y., van West, P., & Diéguez-Uribeondo, J. (2018). Specialized attachment structure of the fish pathogenic oomycete *Saprolegnia parasitica*. *PLoS ONE*, 13(1), e0190361. <https://doi.org/10.1371/journal.pone.0190361>
- Ribot, E., Fair, M., Gautom, R., Cameron, D., Hunter, S., Swaminathan, B., & Barrett, T. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease*, 3(1), 59–67. <https://doi.org/10.1089/fpd.2006.3.59>
- Srinivas, T. N. R., Manasa, P., Begum, Z., Sunil, B., Sailaja, B., Singh, S. K., Prasad, S., & Shivaji, S. (2013). *Iodobacter arcticus* sp. nov., a psychrotolerant bacterium isolated from meltwater stream sediment of an Arctic glacier. *International Journal of Systematic and Evolutionary Microbiology*, 63(Pt_8), 2800–2805. <https://doi.org/10.1099/ijs.0.044776-0>
- Su, W., Zhou, Z., Jiang, F., Chang, X. L., Liu, Y., Wang, S., Kan, W., Xiao, M., Shao, M., Peng, F., & Fang, C. (2013). *Iodobacter limnosediminis* sp. nov., isolated from Arctic lake sediment. *International Journal of Systematic and Evolutionary Microbiology*, 63, 1464–1470. <https://doi.org/10.1099/ijs.0.039982-0>
- Sundberg, L.-R., Ketola, T., Laanto, E., Kinnula, H., Bamford, J. K. H., Penttinen, R., & Mappes, J. (2016). Intensive aquaculture selects for increased virulence and interference competition in bacteria. *Proceedings. Biological Sciences*, 283(1826), 20153069. <https://doi.org/10.1098/rspb.2015.3069>
- Sundell, K., Landor, L., Nicolas, P., Jørgensen, J., Castillo, D., Middelboe, M., Dalsgaard, I., Donati, V. L., Madsen, L., & Wiklund, T. (2019). Phenotypic and genetic predictors of pathogenicity and virulence in *Flavobacterium psychrophilum*. *Frontiers in Microbiology*, 10, 1711. <https://doi.org/10.3389/fmicb.2019.01711>
- Wedekind, C., Gessner, M., Vazquez, F., Maerki, M., & Steiner, D. (2010). Elevated resource availability sufficient to turn opportunistic into virulent fish pathogens. *Ecology*, 91(5), 1251–1256. <https://doi.org/10.1890/09-1067.1>
- Whitman, K. A. (2003). *Finfish and Shellfish Bacteriology Manual: Techniques and Procedures*. Wiley.

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