

ARTICLE

First isolation of *Carnobacterium maltaromaticum* from farmed Rainbow Trout in Virginia

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Abstract

Objective: *Carnobacterium maltaromaticum* is considered an emerging pathogen of salmonids in the United States and around the world.

Methods: Bacterial cultures obtained from the posterior kidney and skin of moribund Rainbow Trout *Oncorhynchus mykiss* from a commercial aquaculture facility in Virginia, USA, grew *C. maltaromaticum*, which was confirmed by additional phenotypic and molecular characterization.

Result: A presumptive diagnosis based on the clinical signs, necropsy observations, histopathology, and bacterial cultures was bacterial septicemia due to *C. maltaromaticum*.

Conclusion: This represents the first documentation of *C. maltaromaticum* in Rainbow Trout from Virginia.

KEYWORDS

Carnobacterium maltaromaticum, emerging pathogen, *Oncorhynchus*, salmonids, trout

INTRODUCTION

Carnobacterium spp. belong to a larger group of lactic acid bacteria that have been described from a variety of food products, including meat, dairy, and seafood. The term “lactic acid bacteria” describes a broad group of gram-positive, catalase-negative, non-spore-forming rods and cocci that are usually nonmotile and that ferment carbohydrates to form lactic acid as the major end product. The current genus name, *Carnobacterium*, was proposed for a group of atypical *Lactobacillus* due to their lack of growth on selective media and the production of oleic acid instead of vaccenic acid as a major cellular fatty acid (Starliper et al. 1992). It is generally also accepted that carnobacteria may be distinguished from typical lactobacilli by their ability to grow at pH 9.0 but not at pH 5.4, by

their growth on acetate agar, and by the presence of meso-diaminopimelic acid in the cell wall peptidoglycan (Ringo and Gatesoupe 1998; Ringo 2008).

Lactobacillus maltaromicus was first described as a lactic acid bacterium isolated from milk that produced a malty-like flavor and aroma (Miller et al. 1974). In 1987, *Lactobacillus piscicola* was renamed as *Carnobacterium piscicola* (Loch et al. 2008). In 1991, the phylogenetic relationships were documented among *Lactobacillus* species, *Carnobacterium* species, and related lactic acid bacteria on the basis of 16S ribosomal DNA sequence data (Collins et al. 1991). Collins et al. (1991) suggested the revision of the taxonomic position of *L. maltaromicus* due to the high 16S ribosomal DNA sequence similarity between this species and *C. piscicola* (Collins et al. 1991), after which Mora et al. (2003) renamed the bacterium as *Carnobacterium maltaromaticum*.

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The taxonomy of lactic acid bacteria has changed considerably during the past few years, and this group presently comprises the following genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Stiles and Holzappel 1997; Gonzalez et al. 2000). It is generally accepted that lactic acid bacteria occur among the normal intestinal flora of fish from the first few days of the fish's life and onwards (Ringo and Gatesoupe 1998; Ringo 2008). Specifically, various *Lactobacillus* and *Carnobacterium* species belong to the normal microbiota of the gastrointestinal tracts of healthy fish (Ringo et al. 2001). However, the population level in the digestive tract is affected by nutritional and environmental factors like diet, stress, and water salinity (Ringo et al. 2001). Some lactic acid bacteria isolated from the gastrointestinal tracts of fish can act as probiotics and perhaps reduce the need for antibiotic use because they colonize the gut and act antagonistically to the growth of gram-negative pathogens (Ringo et al. 2001).

Indeed, *Lactobacillus* spp. have been isolated from a variety of seemingly healthy fish species (Kvasnikov et al. 1977). In several trout species studied in waters around Ontario, Canada, 19% of the fish yielded *Lactobacillus* (Evelyn and McDermott 1961); of those fish, 89% appeared healthy (Evelyn and McDermott 1961; Starliper et al. 1992). In a virulence study conducted with Striped Bass *Morone saxatilis* and Rainbow Trout *Oncorhynchus mykiss*, bacteria of the Striped Bass (01488) and reference strains ATCC 35586 (*L. piscicola*, now reclassified as *C. maltaromaticum*), ATCC 29643 (*Lactobacillus alimentarius*), and ATCC 15434 (*Lactobacillus homohiochii*) were recovered from all of the inoculated fish surviving the challenge, indicating that carrier states can be established in fish populations (Starliper et al. 1992; Toranzo et al. 1993). In a recent comparison examining the genomes of 25 various pathogenic and nonpathogenic strains of *C. maltaromaticum*, only the strains isolated from diseased fish showed *wecC* and *xtmA* genes, and only strains from diseased fish harbored two *wecC* gene paralogs (Roh et al. 2020). These paralogs are known to be involved in the production of D-mannosaminuronic acid, a gram-positive bacterial cell-wall-associated virulence factor of teichuronic acid. Thus, strains of *C. maltaromaticum* that were isolated from diseased fish were distinctly different from strains that were derived from food (e.g., dairy products) in terms of both pathogenicity to fish and the presence of virulence-related genes (Roh et al. 2020).

Hui et al. (1984) undertook a taxonomic study of lactobacilli isolated from salmonids in the U.S. Pacific Northwest and proposed the species *L. piscicola* to

Impact Statement

Clinical signs of *Carnobacterium maltaromaticum* in cultured Rainbow Trout exhibiting anorexia, skin lesions, and abnormal positive buoyancy are described. This is the first documented case of this emerging bacterial disease in trout in Virginia.

accommodate the strains causing the septicemic disease of fish referred to as “pseudo-kidney disease” (Hui et al. 1984; Baya et al. 1991). Further studies indicated that these microorganisms should be included in the *C. piscicola* group (Michel et al. 1986; Baya et al. 1991). However, the histopathologic lesions produced by *C. piscicola* strains in Rainbow Trout and Striped Bass initially caused only mild lesions in the spleen and kidney, and there was no damage to the eyes or musculature (Toranzo et al. 1993).

The *C. piscicola* (PT-31) that was isolated from diseased Rainbow Trout in Spain appeared to be more virulent and pathogenic than other strains of carnobacteria reported previously (Toranzo et al. 1993). Lesions in these Rainbow Trout included bilateral exophthalmia with periorcular hemorrhages; accumulation of ascitic fluid (Cone 1982; Toranzo et al. 1993); and hemorrhages in the liver (Hui et al. 1984; Starliper et al. 1992), swim bladder (Starliper et al. 1992), muscle (Starliper et al. 1992), and intestine (Starliper et al. 1992). The most marked tissue damage, which included acute hemorrhages and necrosis, was observed in the eyes, kidney (Cone 1982), liver (Cone 1982), spleen (Cone 1982), pancreas, and muscle (Hui et al. 1984). Although gram-positive bacilli were observed in all of the organs examined, the highest bacterial numbers were found within the splenocytes, in the necrotic pancreatic tissues (Toranzo et al. 1993), and in blood cavities or blisters under the skin (Herman et al. 1985). Bacterial recovery of the PT-31 isolate was made from affected tissue sites, including the swim bladder, kidney, posterior intestine, ascitic fluid, liver, and spleen from the fish having the most severe clinical disease (Starliper et al. 1992).

Infections with *C. maltaromaticum* have been reported to be more prevalent in spawning *Oncorhynchus* spp. (Hui et al. 1984; Herman et al. 1985; Baya et al. 1991; Mora et al. 2003), with a higher prevalence in females than in males (Herman et al. 1985), and produced gross pathology similar to that of other Carnobacteriaceae species, including increased opacity of the swim bladder. A suggested reason for the female susceptibility may be due to substantial stress from mechanical egg stripping and the retention of dead, unshed eggs in the coelomic cavity, which can act as a site for bacterial growth (Cone 1982). The majority of disease outbreaks associated with *C. maltaromaticum*

have been reported in captive adult Rainbow Trout (Ross and Toth 1974; Cone 1982; Herman et al. 1985; Starliper et al. 1992; Toranzo et al. 1993). Recently, a study reporting the first clinical *C. maltaromaticum* infections in captive *Oncorhynchus* spp. provided strong evidence that infections are widespread in *Oncorhynchus* stocks as well as in hatchery-reared Rainbow Trout (Loch et al. 2011). Despite an apparent susceptibility of females to *C. maltaromaticum* infections, this particular disease epizootic involved an all-male Rainbow Trout captive broodstock population, suggesting that pseudo-kidney disease can ensue if conditions are favorable, regardless of sex (Loch et al. 2011).

The first isolation of a *C. maltaromaticum*-like bacterium from kidneys and swim bladders of Lake Whitefish *Coregonus clupeaformis* caught from Lakes Michigan and Huron was reported by Loch et al. (2008). Except for carbohydrate fermentation, many phenotypic characteristics of the Lake Whitefish isolates coincided with those of *C. maltaromaticum* (Loch et al. 2008). Although a typical pseudo-kidney disease was not observed in the infected Lake Whitefish, the isolation of this bacterium from the kidneys and the gross lesions of splenomegaly, renal and splenic congestion, and thickening of the swim bladder wall with accumulation of a mucoid exudate were consistent with the lesions reported in trout (Loch et al. 2008). In the Loch et al. (2008) study, histologic examination revealed renal and splenic congestion, vacuolation and bile stasis within the liver, epithelial hyperplasia of the swim bladder, fibrin and cellular debris within the lumen of the swim bladder, and neovascularization of the swim bladder.

In our case study, a commercial aquaculture facility with spring-fed, flow-through raceways located in Virginia, USA, experienced an increase in morbidity and mortality of production-sized Rainbow Trout over a 2-week period in January 2018. The water quality parameters were within acceptable ranges, with oxygen levels consistently above 7 mg/L. The fish presented with anorexia and “wasting” (as demonstrated by a concave ventral abdomen), skin lesions, and abnormal positive buoyancy (“floating”).

METHODS

Three production-sized Rainbow Trout with clinical signs were selected and transported live to the Virginia–Maryland College of Veterinary Medicine. Upon arrival, the fish were sedated with sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; 150 mg/L; Syndel) and euthanized by cervical separation; an external examination and necropsy were then performed. Selected tissues from the fish were obtained and placed in buffered 10% formalin for standard histological processing,

hematoxylin and eosin staining, and Gram staining. Bacterial samples obtained from the posterior kidney of all three fish and the skin lesions of two fish were plated on blood agar and brain–heart infusion agar and were cultured at 21°C. Resulting colonies were initially identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analysis. To elucidate the phenotypic and molecular characteristics of the three recovered *C. maltaromaticum* isolates (e.g., M18-123A [posterior kidney], M18-124A [rostral skin lesion], and M18-125A [dorsal skin lesion]), the individual isolates were subjected to additional bacteriological analyses.

Phenotypic characterization

Each isolate was subcultured on trypticase soy agar and incubated at 22°C. After 24–48 h of incubation, oxidase (BD BBL DrySlide; Becton, Dickinson and Company) and catalase (hydrogen peroxide solution, 3%; Millipore Sigma) activities and Gram stain reactions were assessed for the three isolates. Isolates were further characterized for esculin hydrolysis (on bile esculin agar); motility, indole, and hydrogen sulfide production (on sulfur–indole–motility medium); hemolysis (on trypticase soy agar supplemented with sheep's blood); the ability to produce acid from dextrose utilization in the presence and absence of oxygen (final concentration of 1% dextrose in phenol red broth base); acid production from the use of lactose, rhamnose, and inositol (final concentration of 1% of each carbohydrate in phenol red broth base); production of arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase; citrate utilization (on Simmons citrate agar); and assessment of the triple sugar iron reaction (triple sugar iron agar). All tests were incubated at 22°C and read at 72 h; all reagents were purchased from Thermo Scientific unless specified otherwise.

Molecular characterization and phylogenetic analyses

The three isolates were further characterized using two endpoint PCR assays, followed by gene sequencing and phylogenetic analyses. Briefly, nucleic acid extractions were performed using the Qiagen DNeasy Tissue Extraction Kit (Qiagen Sciences) and the manufacturer's protocol for gram-positive bacteria. Extracted nucleic acids were then quantified using a Quant-iT Double-Stranded DNA Assay Kit and a Qubit fluorometer (Life Technologies). Amplification of a partial stretch of the 16S ribosomal RNA (rRNA) gene was conducted via PCR using the assay of Marchesi et al. (1998) and the 27F

(5'-AGA GTT TGA TCM TGG CTC AG-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') primers. Likewise, a partial stretch of the 16S rRNA gene, the intergenic spacer region, and a partial stretch of the 23S rRNA gene were PCR amplified using the PCR assay of Kabadjova et al. (2002) and the 16S-4 (5'-GCT GGA TCA CCT CCT TTC T-3') and 23S-7 (5'-GGT ACT TAG ATG TTT CAG TTC C-3') primers, which produces a characteristic 600-bp band for *C. maltaromaticum* (Pellé et al. 2005; Loch et al. 2008). The PCRs and cycling parameters as well as gel electrophoresis analyses were carried out as previously described (Harrison 2021). Negative controls consisted of nuclease-free water, and a previously confirmed *C. maltaromaticum* isolate served as the positive control. For gene sequence analyses, amplicons ($n = 3$) generated with the 27F–1387R assay were purified and bidirectionally sequenced (Sanger) at Michigan State University's Research Technology Support Facility as described by Harrison (2021). Resultant 16S rRNA gene sequences were quality-trimmed in Chromatogram Explorer Lite version 5.0.2, after which BioEdit version 7.2.5 was used to assemble the contigs. Reference sequences for the type strains of the 12 currently described *Carnobacterium* spp. were downloaded from the National Center for Biotechnology

Information by using BLASTN software (Cailliez-Grimal et al. 2013). The reference sequences and those generated in this study were then aligned using CLUSTAL W in Molecular Evolutionary Genetics Analysis (MEGA) version 10.2.4 (Kumar et al. 2018). Model selection for phylogenetic reconstruction was carried out in MEGA X, whereby the model with the lowest Bayesian information criterion (e.g., Kimura two-parameter model with gamma distribution; Kimura 1980) was selected. For phylogenetic analyses, neighbor-joining analysis was performed in MEGA, and topology robustness was assessed via bootstrap analysis ($n = 1000$ resamplings).

RESULTS

Clinical findings

Externally, most of the Rainbow Trout appeared grossly normal; however, one fish had a superficial, hypopigmented, ulcerative lesion over the dorsum and on the dorsal fin and caudal peduncle, and another fish had a single, superficial, cream-colored ulcerative lesion over the rostrum/nose (Figure 1A–C). In addition, several fish

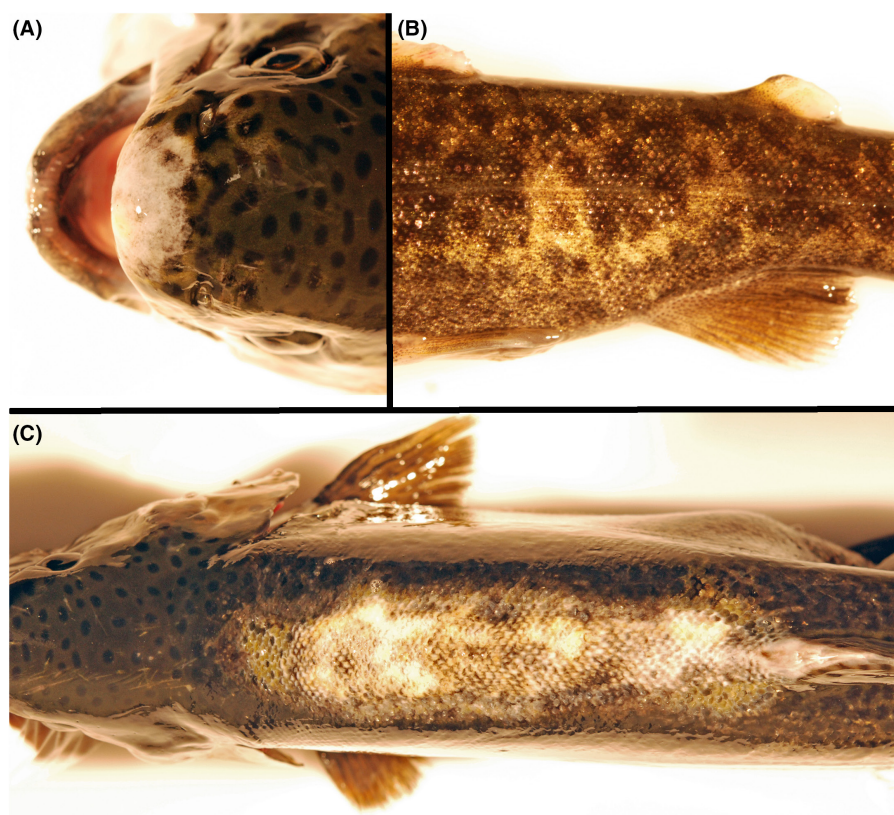


FIGURE 1 Lesions in adult female Rainbow Trout infected with *Carnobacterium maltaromaticum*: (A) superficial ulcerative lesion over the rostrum/nose, (B) hypopigmented lesion over the lateral surface of the caudal peduncle, and (C) hypopigmented lesion over the dorsum.

had unilateral exophthalmia. Wet mount biopsies of the gills did not reveal any obvious bacterial, parasitic, or fungal elements. A skin scrape of the rostrum/nose lesion also did not show any evidence of bacteria, parasites, or fungi. Internally, the fish had a significant amount of fat in the coelomic cavity, although their livers were dark red-brown in coloration. There was no feed in the stomach, cecum, or anterior intestine, but there was a small amount of digested material in the posterior intestine. All three fish were mature, reproductively inactive females. The swim bladders of the fish appeared less translucent than normal, with an increase in the number of superficial blood vessels, but they did not appear enlarged.

Histopathology

There was a diffuse, mild to moderate hyperplasia of the gill epithelium in most of the examined fish, and occasional areas of telangiectasia were scattered throughout the gill tissue. The rostrum/nose lesion consisted of an epithelial ulceration without tissue reaction (Figure 2A). In both the posterior kidney and the anterior kidney, there was a marked increase in melanomacrophages that appeared to be associated with the presence of bacteria (Figure 2B). The ureters and collecting ducts were dilated and contained sloughed epithelial cells. The liver appeared to have normal hepatocytes with no evidence of lipidosis, and there was splenic congestion. Scattered areas of sloughed intestinal epithelium within the lumen of the intestinal tract were present. There was also a significant number of eosinophilic granular cells between the submucosa and muscularis layers of the posterior intestine. There was an increase in opacity with increased storage of adipose tissue in and around the wall of the swim bladder, without

evidence of bacteria on a tissue Gram stain. All other tissues that were examined from these fish appeared normal.

Bacteriology

Bacterial cultures obtained from the posterior kidney of one fish grew *C. maltaromaticum*, while bacterial cultures obtained from the rostrum/nose and skin lesions of the other two fish grew *C. maltaromaticum* and two common environmental species (*Aeromonas sobria* and *Pseudomonas* sp.). The three isolates of *C. maltaromaticum* were uniformly gram-positive, short bacilli without cytochrome oxidase or catalase activities; produced an acid slant over an acid butt without gas or hydrogen sulfide production on triple sugar iron agar; did not utilize citrate, produce acid from rhamnose or inositol, or display lysine decarboxylase or ornithine decarboxylase activities; were nonmotile, were gamma hemolytic, and did not produce indole or hydrogen sulfide on sulfur–indole–motility medium. However, the three isolates hydrolyzed esculin, produced acid in the presence of lactose, showed arginine dihydrolase activity, and produced a malt-like aroma. Collectively, these results are consistent with characteristics of the *C. maltaromaticum* type strain (ATCC 27865; Mora et al. 2003) and *C. maltaromaticum* isolates recovered from other salmonid fish (Loch et al. 2008, 2011).

Using the PCR assay of Kabadjova et al. (2002), all three isolates yielded an approximately 600-bp amplicon, which is considered diagnostic for *C. maltaromaticum* (Pellé et al. 2005; Loch et al. 2008). Likewise, sequence analysis of a partial stretch of the PCR-amplified 16S rRNA gene from each of the three isolates and subsequent phylogenetic analyses dichotomized them into a robustly supported clade also containing

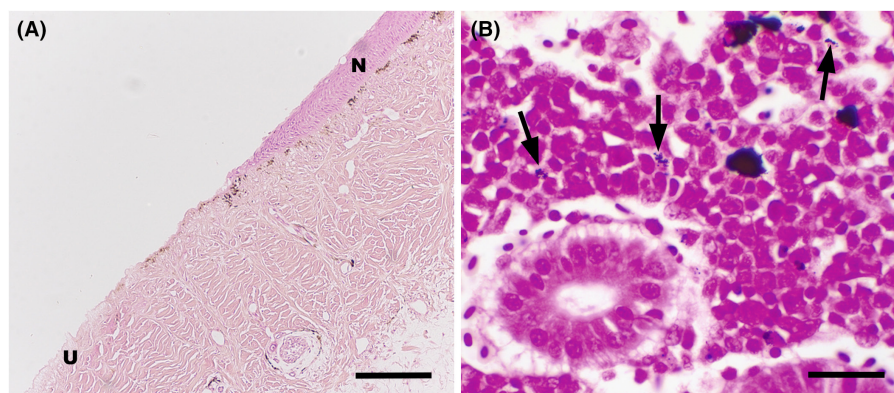


FIGURE 2 (A) Superficial ulcerative lesion over the rostrum/nose of an adult female Rainbow Trout infected with *Carnobacterium maltaromaticum*, showing normal epithelial (N) transitions to ulceration (U) along with the loss of pigmented cells. No bacteria or inflammatory cells were observed in the lesion (hematoxylin and eosin stain; scale bar = 200 μ m). (B) Posterior kidney of an adult female Rainbow Trout exhibits colonies of gram-positive bacteria (arrows) without inflammation or necrosis (Gram stain; scale bar = 50 μ m).

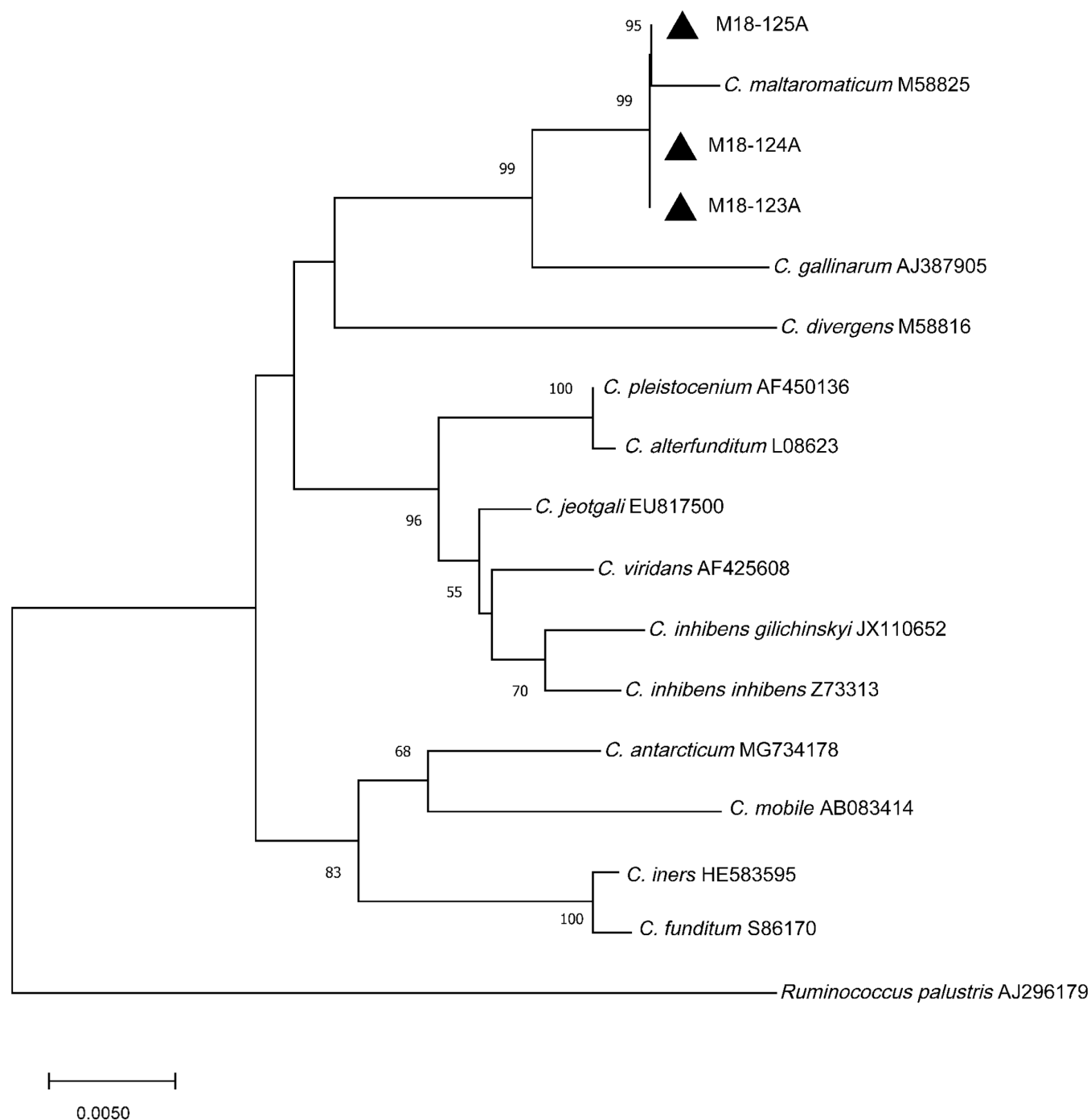


FIGURE 3 A dendrogram (constructed in Molecular Evolutionary Genetics Analysis X) depicting the relationships of the three bacterial isolates recovered from diseased Rainbow Trout in this study (denoted by black triangles) with reference 16S ribosomal RNA gene sequences derived from the type strains of all currently described *Carnobacterium* spp. The relationships were inferred using the neighbor-joining method (Saitou and Nei 1987), and the percentage of replicate dendrograms showing the depicted clustering of taxa is presented at each node (1000 bootstrap replicates; only bootstrap values >50% are displayed). Evolutionary distances were estimated using the Kimura two-parameter method (Kimura 1980), whereby rate variation among sites was modeled with a gamma distribution. In total, 1169 positions were evaluated in the depicted data set using the complete deletion option. *Ruminococcus palustris* served as the outgroup.

the *C. maltaromaticum* type strain (Figure 3). However, some sequence variation between the type strain and the *C. maltaromaticum* isolates recovered in this study was evident (Figure 3).

DISCUSSION

We report the isolation and identification of the presumptive causative agent of pseudo-kidney disease, *C.*

maltaromaticum, from a mortality event in 14-month-old, farmed Rainbow Trout in Virginia. Phenotypic, molecular, and phylogenetic analyses not only showed strong agreement on the identification of this bacterium but also were consistent with results for the *C. maltaromaticum* type strain (ATCC 27865) and other isolates recovered from fish in the genera *Oncorhynchus* and *Coregonus* (Loch et al. 2011). *Carnobacterium* spp. infections in fish in the United States have been increasingly reported and include the following species: Rainbow Trout (Cone 1982; Hui et al. 1984; Herman et al. 1985; Starliper et al. 1992), Coho Salmon *O. kisutch* (Starliper et al. 1992), Chinook Salmon *O. tshawytscha* (Baya et al. 1991), Brown Trout *Salmo trutta* (Baya et al. 1991), Cutthroat Trout *O. clarkii* (Hui et al. 1984), carp (Mora et al. 2003), Striped Bass (Baya et al. 1991), Channel Catfish *Ictalurus punctatus* (Baya et al. 1991), Lake Whitefish (Loch et al. 2008, 2011), and sharks (Steele et al. 2019). This infection has been reported in U.S. states including Idaho (Starliper et al. 1992), Michigan (Loch et al. 2008), Oregon (Hui et al. 1984), and West Virginia (Gonzalez et al. 2000) as well as in the Chesapeake Bay (Baya et al. 1991). This represents the first report of *C. maltaromaticum* in cultured Rainbow Trout from Virginia.

Findings from this study contribute to a growing body of evidence that *C. maltaromaticum* should be considered an emerging pathogen of salmonids, especially since it has been detected from wild, feral, and farmed salmonid fish. Loch et al. (2008) considered this organism to be an emerging pathogen of salmonids in the Great Lakes region. In addition, *C. maltaromaticum* has been linked to increasing reports of disease outbreaks around the world.

ACKNOWLEDGMENTS

There are no acknowledgments.

CONFLICTS OF INTEREST

The authors state that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

There is no data available for this diagnostic case.

ETHICS STATEMENT

The animals in this study were humanely euthanized following the 2020 Guidelines for the Euthanasia of Animals.

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