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Experimental Challenge of Chinook Salmon (*Oncorhynchus tshawytscha*) With *Tenacibaculum maritimum* and *Tenacibaculum dicentrarchi* Fulfils Koch's Postulates

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ABSTRACT

The bacterial skin disease tenacibaculosis, caused by *Tenacibaculum* species, affects numerous economically important marine fish, including salmonids. This study reports the ability of three *Tenacibaculum maritimum* strains, belonging to different molecular O-AGC types, and a single *Tenacibaculum dicentrarchi* strain to induce tenacibaculosis in farmed Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum 1792) in Aotearoa New Zealand. Naïve Chinook salmon were exposed to *T. maritimum* (2×10^8 cells/mL) and *T. dicentrarchi* (2×10^7 cells/mL) by immersion using natural seawater. Clinical signs of tenacibaculosis were apparent in all *T. maritimum* strains used in the challenged fish. Of these, 100% of the fish challenged with O-AGC Type 2-1 and Type 3-2 strains became moribund, whereas only 60% of the O-AGC Type 3-0 challenged fish became moribund. Fish exposed to *T. dicentrarchi* showed more severe symptoms, exposing musculature in 51% of the challenged population, with 28% of fish becoming moribund. Gross pathological signs of fin rot, scale loss, skin ulcers and haemorrhagic skin spots were observed for both *Tenacibaculum* species and were consistent with those observed on farmed fish. Pure *T. maritimum* and *T. dicentrarchi* cultures were reisolated from epidermal damage of challenged fish. *Tenacibaculum* species was not isolated from the anterior kidney of affected fish, which indicates no systemic infection in Chinook salmon.

1 | Introduction

Salmon are the highest value marine fish species in the global aquaculture sector (FAO 2022; Global Salmon Initiative 2023). Since the 1980s, salmonid aquaculture has been hampered by tenacibaculosis (formerly known as marine flexibacteriosis), an economically significant marine infectious disease (Boerlage et al. 2020; Handlinger, Soltani, and Percival 1997; Lagadec et al. 2021; Spilsberg et al. 2022; van Gelderen, Carson, and Nowak 2011). Tenacibaculosis is an epidermal infection caused by the biofilm-producing, Gram-negative obligate marine bacteria *Tenacibaculum* spp. on external mucosal surfaces of fish including the skin, fins, gills, palate and teeth (Frisch et al. 2018; Jones and Madsen 2019; Levipan et al. 2019; Nowlan, Lumsden,

and Russell 2020; Småge et al. 2017). *Tenacibaculum* species that are known to cause disease or associated with tenacibaculosis in marine fish include *Tenacibaculum maritimum* (see Avendaño-Herrera, Toranzo, and Magariños 2006a, 2006b; Frisch et al. 2018; Mabrok et al. 2022; Nowlan et al. 2021; Ostland, Morrison, and Ferguson 1999; van Gelderen, Carson, and Nowak 2011), *Tenacibaculum dicentrarchi* (see Klakegg et al. 2019; Nowlan et al. 2021), *Tenacibaculum soleae* (see López et al. 2010; Piñeiro-Vidal et al. 2008a), *Tenacibaculum discolor* (see Piñeiro-Vidal, Riaza, and Santos 2008b), *Tenacibaculum piscium* (see Avendaño-Herrera et al. 2022), *Tenacibaculum bernardetii* (see Avendaño-Herrera, Saldarriaga-Córdoba, and Irgang 2023) and *Tenacibaculum finnmarkense* (see Avendaño-Herrera et al. 2020; Småge et al. 2018). While Atlantic salmon (*Salmo salar*) aquaculture has been impacted by tenacibaculosis since the 1990s (Avendaño-Herrera, Toranzo, and Magariños 2006a, 2006b; Ostland, Morrison, and Ferguson 1999), farmed Chinook salmon (*Oncorhynchus tshawytscha*) was considered to be resistant to *T. maritimum* in the Pacific Northwest (Kent and Poppe 2002). No major tenacibaculosis outbreaks have been reported even though *T. maritimum* was initially detected in Chinook salmon in marine pens in California in the early 1990s (Chen, Henry-Ford, and Groff 1995). More recently, Bass et al. (2022) hypothesised that *T. maritimum* was associated with low survival and poor body condition in wild Chinook and Coho salmon (*Oncorhynchus kisutch*) in British Columbia sampled early in their marine life.

Tenacibaculum maritimum (formerly *Flexibacter maritimus*; Wakabayashi, Hikida, and Masumura 1986) was first reported in Aotearoa New Zealand Chinook salmon in 1989 (Boustead 1989, 87p.). At the time, it was found in association with skin lesions and significant levels of mortality of juvenile salmon following transfer to marine waters. Since 2012, Chinook salmon farms located in the Marlborough Sounds have consistently reported elevated levels of mortality associated with skin disease. Disease investigations from 2015 to 2019 of fish exhibiting skin ulcers showed inconsistent detection of *T. maritimum* and/or *Rickettsia*-like organisms by polymerase chain reaction (PCR), indicating a multi-agent disease condition (Brosnahan et al. 2019; Chetty et al. 2023; Fischer and Appleby 2015). However, in the summer of 2020, a field study, using culture methods, revealed 100% association of *T. maritimum* in clinically compromised fish with skin lesions (Kumanan et al. 2022) and no detection of New Zealand *Rickettsia*-like organisms (NZ-RLO1 and NZ-RLO2) by PCR (unpublished data). There were also incidences of *T. maritimum* co-infection with *T. dicentrarchi* or *T. soleae* (see Kumanan et al. 2022). Further analyses revealed that the *T. maritimum* associated with the mortality event represented strains from three different molecular O-antigen gene cluster (O-AGC) types (O-AGC Type 3-0, Type 2-1 and Type 3-2) (Kumanan et al. 2024). Consequently, tenacibaculosis was categorised as a priority disease by the Chinook salmon industry in Aotearoa New Zealand.

The host–pathogen relationship and pathogenesis of tenacibaculosis in Chinook salmon has scarcely been studied. Proving causality of a disease in uncontrolled aquatic environments can be complex due to potential multifactor influences (i.e., increasing sea surface temperatures and multiple disease agents). In the case of salmon skin disease and mortalities in Aotearoa New Zealand, the scenario is particularly complex given the identification of multiple contributing factors including bacterial pathogens (NZ RLOs, *Tenacibaculum* spp. and *Vibrio* spp.) and environmental factors such as warming seawater, harmful algae and biofouling organisms (Fletcher et al. 2023; Lane, Brosnahan, and Poulin 2022; Rolton et al. 2022). The Henle–Koch postulates (Evans 1976; Koch 1884), commonly called Koch's postulates, have been successfully applied to several aquatic diseases to prove causality of disease (see Hutson et al. 2023 for a review). While the causality of tenacibaculosis by *T. maritimum* and *T. dicentrarchi* has been studied in Atlantic salmon (Avendano-Herrera et al. 2016; Frisch et al. 2018; Klakegg et al. 2019; Nowlan et al. 2021; van Gelderen, Carson, and Nowak 2011), the susceptibility of Chinook salmon to tenacibaculosis has not been demonstrated experimentally.

The aim of this study was to establish whether *T. dicentrarchi* strain and *T. maritimum* strains from three different O-AGC types, isolated from Aotearoa New Zealand Chinook salmon, can cause skin disease or mortality in Chinook salmon in fulfilment of Koch's postulates. A second aim was to investigate whether *Tenacibaculum* species can proliferate within internal organs of Chinook salmon and cause septicaemia. Several published studies have suggested that *T. maritimum* can cause systemic infections (Avendaño-Herrera, Toranzo, and Magariños 2006a, 2006b; Faílde et al. 2013; Frisch et al. 2018; Nowlan et al. 2021). To determine whether *T. maritimum* was able to cause septicaemia in Chinook salmon, we analysed fish with clinical tenacibaculosis from both farm settings and challenge trials to assess the presence of viable *T. maritimum* in the kidney of affected Chinook salmon.

2 | Materials and Methods

2.1 | Animal Ethics Statement

All procedures performed involving live salmon were carried out in accordance with New Zealand government regulations and associated ethical standards as administered by the Nelson Marlborough Institute of Technology Limited Animal Ethics Committee approval (Ref. AEC2018 CAW011 and AEC-2022-CAW-02).

2.2 | Source of Fish and Husbandry

Juvenile Chinook salmon (*O. tshawytscha*) were sourced from a freshwater hatchery at Tentburn, New Zealand. We used two different sizes of salmon (Table 1) due to a small pool of fish available that had not been immunised with a *T. maritimum* vaccine at the hatchery. Salmon were transported in fresh water in a custom-built transport vehicle with oxygen control, off-gassing and real-time monitoring. The salmon were housed in 1000 L tanks at *Te Wero Aro-anamata*, Cawthron's aquatic physical biocontainment (PC2) facility. Each tank was equipped with its own recirculating aquaculture system (RAS), which consisted of temperature control ±0.3°C through a supervisory control and data acquisition system, real-time alarmed oxygen monitoring, particle filtration with bubble bead filters, inline ultraviolet (UV) disinfection at 56 mj/cm^2 dose and 50 L/min water flow, foam fractionator for

TABLE 1 | *Tenacibaculum* species and strains used in the Chinook salmon experimental challenge trials, indicating bacterial concentration used in each challenge. Initial bacterial density in fresh broth = bacterial density obtained post incubation; adjusted dose cell/mL in bath challenge = bacterial density used in the challenge bath; Exp = Experiment.

Exp	Bacterial species and O-AGC types	Year of isolation	Adjusted concentration (cells/mL) in bath challenge (direct count)	Concentration (CFU/mL) in bath challenge (viable count)	Average fish weight (g) ± standard error of mean
1	*Tenacibaculum maritimum* (CCCM20/006, O-AGC Type 3-0)	2020	2×10^8	1.9×10^8	223 ± 4.5
2	*Tenacibaculum maritimum* (CCCM20/133, O-AGC Type 2-1)	2021	2×10^8	2.0×10^8	120 ± 1.2
3	*Tenacibaculum maritimum* (CCCM20/102, O-AGC Type 3-2)	2020	2×10^8	1.9×10^8	120 ± 1.2
4	*Tenacibaculum dicentrarchi* (CCCM21/136)	2021	2×10^7	2.1×10^7	120 ± 1.2

fine particle removal and a biofilter. Natural seawater used in this study was sourced from the Cawthron Aquaculture Park, Glenduan, Nelson, New Zealand, and was UV treated and filtered to 5 μm prior to use. On arrival, the fish were transferred into water at 17°C and 15 ppt salinity and were then gradually acclimated to 35 ppt water salinity over 16 days. The fish were fed to satiation three times per day. Fish were not fed for 2 days prior to manipulation or for the remainder of the experiment post challenge to avoid water quality differences among the treatments. We also sought to avoid further husbandry stress to the fish, which occurs from remnant feed removal using a vacuum method and bacterial replication due to organic load build-up from uneaten fish feed.

2.3 | Bacterial Strain and Propagation

The *T. maritimum* strains (CCCM20/006, CCCM20/102 and CCCM20/133), belonging to three O-AGC types, and a single *T. dicentrarchi* strain (CCCM21/136) used in this study were isolated from skin ulcers of moribund Chinook salmon during disease outbreaks in the Marlborough Sounds, Aotearoa New Zealand (Table 1). The strains were confirmed to be *T. maritimum* and *T. dicentrarchi* by species-specific PCR (von Ammon et al. 2022; Wilson, Douglas, and Dunn 2019). The strains were stored at −80°C on cryobeads (Microbeads) and revived on Marine Shieh's Medium agar (MSM; Wilson, Douglas, and Dunn 2019) and ZoBell's Marine Agar 2216E (MA) and then incubated at 22°C for 48 h. Growth of *T. maritimum* on both types of media was assessed and purity was checked by Gram stain. Pure colonies were inoculated into 50 mL primary MSM and marine broth (MB), respectively, in 150 mL baffled conical flasks and incubated at 22°C for 48 h at 180 rpm on a Ratek orbital shaker. Primary broths were used to inoculate 1 L MSM and MB for the challenge trial and incubated as above. When bacterial aggregates were observed, only the homogeneous liquid phase of bacterial broth was used for the cell count and experiment. An approximate cell density was assessed by microscopy using a Helber bacteria counting chamber. To assess cell viability by plate count, 100 μL of 10-fold dilution of broth culture was inoculated onto MSM using a spread plate method and incubated at 22°C for 48 h. Colony-forming units (CFUs) were enumerated to determine viable cell concentration in the immersion challenge.

2.4 | Pre-trial Health Check of Naïve Fish and Tank Water Bacteriology

The mean weight of experimental fish used in the *T. maritimum* and *T. dicentrarchi* infection challenges are as shown in Table 1. Five fish per tank were euthanised prior to sampling by the percussive stunning. These fish were sampled for bacteriology for potential pathogenic bacterial species such as *T. maritimum* and *T. dicentrarchi* and for molecular analysis to screen for NZ-RLO1, NZ-RLO2 and *Yersinia ruckeri*. Skin mucus and gill swab were collected for each fish using a sterile swab and directly streaked onto Marine Shieh's Selective Medium (MSSM) (Wilson, Douglas, and Dunn 2019), thiosulfate–citrate–bile salts–sucrose agar (TCBS), MA and Columbia sheep's blood agar (5% blood and 0.5% NaCl) (BA). The fish were aseptically dissected to expose the internal organs. Using sterile forceps and a scalpel blade, the digestive system was removed, without puncturing the digestive content or the heart, to expose the anterior kidney. The epithelial membrane covering the anterior kidney was removed using a

sterile scalpel blade and a sterile swab was inserted directly into the anterior kidney, avoiding contact with any other internal organs. The swab was directly inoculated onto all four media as described. All inoculated media were incubated at 22°C for 72 h. Different sets of sterile tools were used for each fish sampled to avoid cross-contamination of the anterior kidney with external microbiota. Approximately 3 mm^3 of skin, gill and anterior kidney tissue were aseptically dissected and stored in DNA/RNA Shield (Zymo Research) at 4°C for molecular analysis. A tetraplex assay was employed to detect and quantify the four primary pathogens of Aotearoa New Zealand Chinook salmon following von Ammon et al. (2022). Fish holding RAS tank water was tested for the presence/absence of *Tenacibaculum* species by plating 100 µL of water onto MSSM and incubated at 22°C for 72 h.

2.5 | Experimental Infection of *T. maritimum* and *T. dicentrarchi* by Immersion

Tenacibaculum maritimum strains (Type 3-0: CCCM20/006; Type 2-1: CCCM20/102; and Type 3-2: CCCM20/133) and *T. dicentrarchi* strain (CCCM21/136) grown in MSM broth were used for separate immersion challenges at a fish density of 60 kg/m^3 in the immersion bath for an hour. Immersion density was determined based on two scenarios, one being the transportation tank density, where fish are transferred from the hatchery to the farm at tank densities reaching up to 85 kg/m^3 for at least 5–10 h. Second, fish in sea pens are graded for size during production to ensure uniform growth. This process involves crowding the fish in a smaller area and passing them through a grading bar. Scale loss and skin abrasion may occur during this process due to crowding and physical contact with grading equipment. We took this into account in our experimental design, in an attempt to replicate the stressors fish encounter during production (Masud, Ellison, and Cable 2019). For each trial, two experimental groups (control and treatment) were tested in triplicate (Figure 1). Control ($n = 30$ fish) and treatment groups ($n = 30$ fish) were transferred by net to individual 100 L polypropylene fish bins containing 17°C UV–treated natural seawater at 35 ppt salinity. Oxygen (100%–110% saturation) was supplied to each challenge bath during the pathogen challenge. The control and treatment groups were challenged simultaneously. The bacterial concentration used for the experiment was based on *Tenacibaculum* counts observed in net cleaning effluent in summer and pathogenicity tests performed on a Chinook salmon skin cell line (data not shown). The bacterial concentration in the immersion bath was standardised to 2×10^8 cells/mL for all three O-AGC types of *T. maritimum* and 2×10^7 cells/mL of *T. dicentrarchi*, as described by Nowlan et al. (2021). *T. dicentrarchi* bacterial density in the challenge bath was tested at 2×10^7 cells/mL due to the lower bacterial yield in broth culture than that achieved for *T. maritimum*. The same volume of sterile MSM broth was added to UV–treated seawater in the control immersion bath. A lid was placed on top of the bin to minimise splash fall-out and fish were held immersed for 60 min. Fish from the treatment and control groups were redistributed to individual triplicate RAS tanks stocked with 10 fish/1000 L tank (1.2–2.2 kg/m^3) for *T. maritimum* challenges and 13 fish/1000 L tank (1.6 kg/m^3) for the *T. dicentrarchi* challenge. Fish were visually monitored every 3 h for clinical and behavioural signs of disease, such as scale loss, skin lesions, skin ulcers, fin erosions, epidermal haemorrhage, exophthalmia and mouth erosion.

2.6 | Post-Mortem and End of Trial Examination

Taking fish welfare into consideration, as we observed disease progression, the *T. maritimum* O-AGC Type 3 and *T. dicentrarchi* challenge experiments were terminated at 8 and 11 days post-infection (DPI), respectively. *T. maritimum* trials of strain O-AGC Type 2-1 and Type 3-2 were ended at 3 DPI. Throughout the experiment, fish found moribund or dead were immediately removed for necropsy. We found that percussive stunning method used for euthanasia during the pre-trial health assessment induced lamellar aneurysm. To avoid this artefact in histological interpretation, moribund or survivors were euthanised by iki-jime method (Diggles 2015; Mitchell et al. 2023). Fish from the control group were euthanised and sampled first, followed by the treatment group, to prevent contamination.

2.6.1 | Bacteriological Analysis

Fish were placed sample side up (side presenting clinical disease), and sampled only on one side, to avoid contamination from the supporting surface. Bacterial swab samples were collected from external lesions and the anterior kidney. Bacterial swab samples collected from the skin, mouth, gill or tail lesions were inoculated onto MSSM to reisolate the organism used in the challenge. To rule out the presence of other pathogens that cause skin disease, samples were also inoculated on TCBS, BA and MA, and incubated as described in Section 2.4 for a minimum of 4 days. After incubation, bacterial colony morphology consistent with *T. maritimum* and *T. dicentrarchi* was assessed on MSSM and MA (see figure 2 in Kumanan et al. 2022). Species identification of bacterial colonies was done using species-specific primers as described in Section 2.6.3 and the O-AGC types of *T. maritimum* were confirmed by end-point PCR using primers developed for O-AGC–based serotyping as described in Lopez et al. (2022a). In addition, to investigate whether *T. maritimum* or *T. dicentrarchi* are viable systemically, a swab from the anterior kidney was plated onto all four media types as described in Section 2.4. Viable *Tenacibaculum* counts in RAS tank water were quantified daily for all trials from 1 DPI to the end of a trial for each *Tenacibaculum* species/strain. One hundred microlitres of 10-fold diluted RAS tank water was plated onto MSSM in triplicate and incubated at 22°C for 72 h.

2.6.2 | Histopathology

Histopathological analysis was carried out on moribund fish challenged with *T. maritimum* (O-AGC Type 3-0, $n = 7$) and *T. dicentrarchi* ($n = 3$) and control ($n = 3$) trials. Approximately 5 mm^3 of skin tissue (periphery of the lesion), gill and anterior kidney were excised and preserved in 1:10 parts of

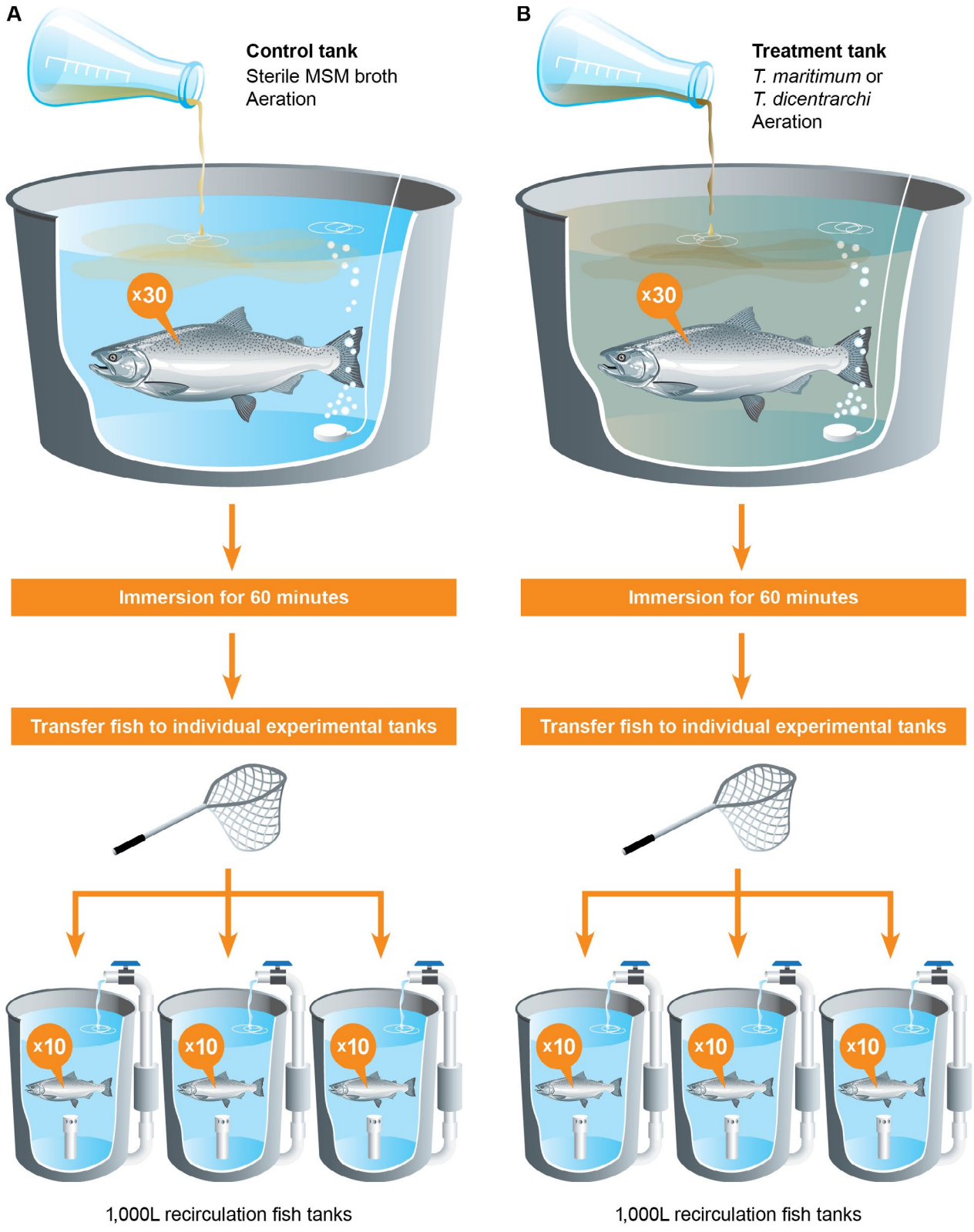

FIGURE 1 | Schematic representation of the in vivo immersion challenge of Chinook salmon in individual recirculating aquaculture systems (RAS). Figure A represents the control group and Figure B represents the challenged group with three O-AGC Types of *Tenacibaculum maritimum* strains (Type 3-0: CCCM20/006; Type 2-1: CCCM20/102; and Type 3-2: CCCM20/133) and *Tenacibaculum dicentrarchi* (CCCM21/136, where 13 fish were used instead of 10).

10% neutral buffered formalin. Tissue samples were sent to Gribbles Veterinary Pathology, Christchurch, New Zealand for processing, paraffin embedding and haematoxylin and eosin (H & E) staining and the histopathological examination was performed at Cawthron Institute to evaluate the presence or absence of pathology.

2.6.3 | Droplet Digital PCR (ddPCR) Analysis of Infected Fish

Tissue samples of fish from *T. maritimum* O-AGC Type 3-0 (control $n = 30$ and challenge $n = 29$, one fish was excluded due to bleach contamination) and from *T. dicentrarchi* (control $n = 15$, challenge $n = 15$) challenge trials were analysed to investigate the presence of DNA from pathogens in various organs of infected fish. Approximately 30 mg of individual fish tissue (skin, gill and anterior kidney) from diseased fish was used for genomic DNA (gDNA) extraction using Quick-DNA Miniprep Plus Kit (Zymo Research) following the manufacturer's instructions. Tissues from control fish were processed prior to samples from the treatment group and each tissue type was processed on separate days to avoid potential cross-contamination during DNA extraction. One hundred nanograms of gDNA from each tissue type or strain was used to perform ddPCR assay using a QX200 Droplet Digital PCR System (Bio-Rad, Hercules, USA). For *T. maritimum* detection, primer targeting a 155 bp region gene of the 16S rRNA (forward, 5′-TGCCTTCTACAGAGGGATAGCC-3′; reverse, 5′-CTATCGTTGCCATGGTAAGCCG-3′) and probe (5′-HEX-CACTTTGG AATGGCATCG-BHQ1-3′) (Fringuelli et al. 2012) were used as described by von Ammon et al. (2022).

Tenacibaculum dicentrarchi–specific primers targeting a 688 bp region of 16S rRNA (forward, 5′-AAT GTA GTG CTT CGG CAT C-3′; reverse, 5′-TCA CTG AAC CGA AGT CC-3′) (Wilson, Douglas, and Dunn 2019) were used for ddPCR to quantify *T. dicentrarchi* DNA. These *T. dicentrarchi* primers were transferred to ddPCR including the validation of cross-reactivity with non-target DNA (*T. maritimum* and *Vibrio* sp.). Only *T. dicentrarchi* DNA amplified as positive (data not shown). Positive and negative controls were included for each run afterwards. Each ddPCR reaction tube consisted of 1 μL of each forward and reverse primer (10 μM), 10 μL of 2× QX200 ddPCR EvaGreen Supermix (BioRad, Hercules, CA, USA), 8 μL of PCR grade water and 1 μL of DNA template. The thermocycling conditions were as follows: 2 min denaturation at 94°C, 36 cycles of denaturation (10 s at 94°C), annealing (15 s at 60°C) and extension (60 s at 72°C) and final extensions for 2 min at 72°C.

2.6.4 | Viability of *T. maritimum* in External and Internal Organs of Affected Fish

To test our hypothesis that *T. maritimum* does not survive and replicate systemically, we utilised moribund Chinook salmon from commercial farm sites in the Marlborough Sounds, South Island (Otanerau, $n = 28$, Waihinau, $n = 8$ and Kopāua, $n = 5$; Table 2). These fish were vaccinated against *T. maritimum*. All fish exhibited various clinical signs of tenacibaculosis including scale loss, skin ulcers, erythematous skin lesion and fin and mouth erosion. We used a bacteriological method to detect the presence or absence of viable *T. maritimum* and compared it against positive ddPCR detection, which amplifies nucleic acid from both viable and non-viable cells. A swab sample from the skin and anterior kidney was inoculated onto MSSM to compare the presence of viable *T. maritimum* between external and internal tissue as described in Section 2.4. Molecular analysis of *T. maritimum* was conducted on tissues from skin and anterior kidney as described in Section 2.6.3.

2.7 | Statistical Analysis

2.7.1 | Kaplan–Meier Survival Curve

R-Studio Version 4.2.1 (R Core Team 2020) was used for all statistical analyses. Survival of Chinook salmon in each experimental group was plotted using the Kaplan–Meier survival curve with the R package 'survival' and applying the daily morbidity data.

2.7.2 | Statistical Analysis of Bacterial Load and Correlation With Health Status

Differences in ddPCR detection of *T. maritimum* O-AGC Type 3 and *T. dicentrarchi* in copies/μL and square root transformed for the factor 'tissue' (skin, gill and anterior kidney) and the second factor 'health status' (mortality, moribund and survivors) were analysed within the R Project statistical software (R Core Team 2020). The non-parametric two-factorial Scheirer–Ray–Hare tests were undertaken, followed by post hoc pairwise Wilcoxon comparisons, considering results as significant if $p \leq 0.05$. Survival in the control groups was 100% (unaffected); therefore, these were excluded from statistical analysis. In addition, linear regression curves were plotted to compare bacterial loads (copies/μL) between the anterior kidney and the combined external tissues (skin and gill) for *T. maritimum* and *T. dicentrarchi*, respectively. A test for correlation (using Pearson's correlation coefficient) was used to test for significance.

3 | Results

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.1 | Bacterial Strain and Propagation and Pre-trial Assessment

Three *T. maritimum* strains (O-AGC Type 3-0, Type 2-1 and Type 3-2) revived from microbeads performed consistently better on MSM agar and in broth compared to Zobell's Marine Agar 2216E and broth. MSM resulted in higher cell densities, less aggregation and less biofilm production (Figure S1).

Tenacibaculum dicentrarchi did not form aggregates in either medium, and the cell density in MSM broth was higher compared to MB. *Tenacibaculum maritimum* and *T. dicentrarchi* cultured in MSM broth with vigorous shaking at 180 rpm resulted in a yield of 10^9 and 10^8 cells/mL, respectively, by direct count using the Helber counting chamber (Table 1). Bacterial concentration by direct count for strains of *T. maritimum* and *T. dicentrarchi* were then adjusted to 2×10^8 and 2×10^7 cells/mL, respectively, for the bath challenge. However, the viability assessment performed on the final challenge bath showed ±10% variation in the viable bacterial concentration in comparison to direct cell count (Table 1).

The pre-trial fish health assessment confirmed that the fish were in optimal health at the time of challenge and free from all the tested pathogens. Bacterial analysis conducted on tank water confirmed that there were no pre-existing *Tenacibaculum* species in the RAS.

TABLE 2 | Viable and non-viable *Tenacibaculum maritimum* detection in skin and anterior kidney by bacterial culture and ddPCR in naturally infected Chinook salmon, all with skin lesions, from farm outbreaks. NG = no growth of *T. maritimum*, yes = growth of *T. maritimum*.

		Skin abnormalities		Anterior kidney	
Location	**Fish ID**	**Bacterial culture**	**ddPCR (copies/μL)**	**Bacterial culture**	**ddPCR (copies/μL)**
Otanerau	1	Yes	0.769	NG	0
Otanerau	2	Yes	284	NG	0
Otanerau	3	NG	0	NG	0
Otanerau	4	Yes	1.34	NG	0
Otanerau	5	Yes	575	NG	0
Otanerau	6	Yes	102	NG	0
Otanerau	7	NG	2.03	NG	0
Otanerau	8	NG	0	NG	0
Otanerau	9	NG	0	NG	0.103
Otanerau	10	NG	0	NG	0
Otanerau	11	Yes	215	NG	0
Otanerau	12	NG	1.44	NG	0
Otanerau	13	NG	0	NG	0
Otanerau	14	Yes	0.05	NG	0
Otanerau	15	NG	0.459	NG	0.429
Otanerau	16	Yes	0.06	NG	0.09
Otanerau	17	NG	2.77	NG	0
Otanerau	18	Yes	199	NG	0
Otanerau	19	Yes	25.8	NG	0
Otanerau	20	NG	0	NG	0.109
Otanerau	21	NG	0	NG	0
Otanerau	22	Yes	186	NG	0
Otanerau	23	Yes	5.56	NG	0
Otanerau	24	Yes	2.9	NG	0
Otanerau	25	NG	0	NG	0
Otanerau	26	NG	0	NG	0
Otanerau	27	Yes	0.172	NG	0
Otanerau	28	Yes	835	NG	0
Kopāua	29	Yes	1,000,000	NG	0
Kopāua	30	Yes	1373	NG	0
Kopāua	31	Yes	545	NG	0
Kopāua	32	Yes	2.3	NG	0
Kopāua	33	Yes	0.878	NG	0
Waihinau	34	Yes	1,000,000	NG	0
Waihinau	35	Yes	1789	NG	0
Waihinau	36	Yes	1295	NG	0

(Continues)

TABLE 2 | (Continued)

Location	Fish ID	Skin abnormalities		Anterior kidney	
		Bacterial culture	ddPCR (copies/μL)	Bacterial culture	ddPCR (copies/μL)
Waihinau	37	Yes	530	NG	0
Waihinau	38	Yes	513	NG	0
Waihinau	39	Yes	461	NG	0
Waihinau	40	Yes	134	NG	0
Waihinau	41	Yes	7.26	NG	0

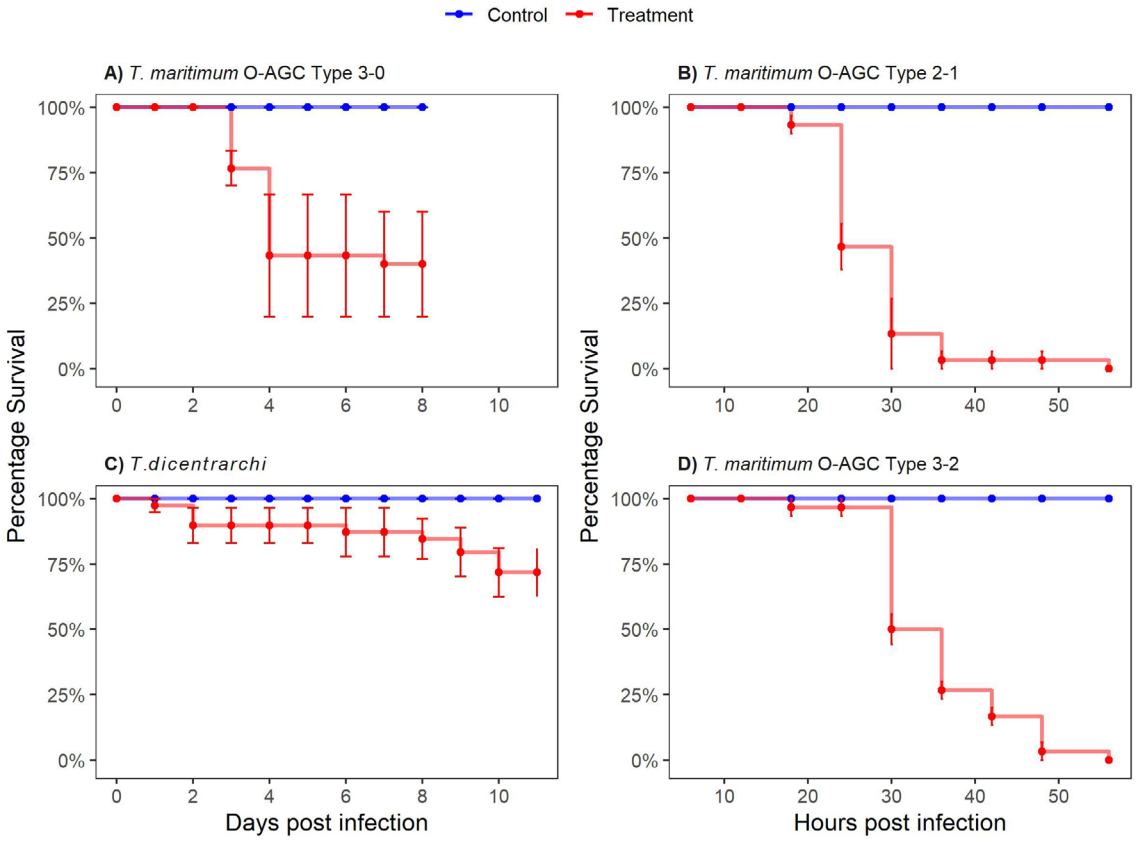

FIGURE 2 | Kaplan–Meier survival analysis of naïve Chinook salmon *Oncorhynchus tshawytscha* experimentally challenged with three molecular O-AGC type strains of *T. maritimum* (A, B and D) and an isolate of *T. dicentrarchi* (C).

3.2 | Clinical Signs and Gross Pathology

3.2.1 | *Tenacibaculum maritimum* Challenge

On 1 DPI, fish challenged with *T. maritimum* strains, belonging to O-AGC Type 3-0, Type 2-1 and Type 3-2 in independent trials, were observed to have a reduced swimming rate and displayed white lesions on their abdomen. On close observation, it was confirmed that the patches were scales protruding from the epidermis. At 2 DPI in the *T. maritimum* O-AGC Type 3-0 challenge, erratic swimming and loss of equilibrium was observed in moribund fish and the first mortality occurred. Increased opercular activity was common and the cumulative survival of Chinook salmon in the O-AGC Type 3-0 treatment group at 8 DPI was 40% ± 20% (Figure 2A). On the other hand, all smaller fish challenged with strains of O-AGC Type 2-1 and Type 3-2 became moribund within 60 h after challenge (Figure 2B,C).

Gross pathology signs consistent with tenacibaculosis (including morbidity and mortality) were observed in all fish exposed to the three *T. maritimum* O-AGC type strains. Affected fish exhibited varying degrees of pathology ranging from scale loss, skin ulcers, erythematous skin lesion, pectoral and tail fin necrosis, mouth erosion, gill erosion, ventral haemorrhaging and exophthalmia (Figure 3). No consistent pathologies were observed in the internal organs of experimental fish except for one surviving fish from the *T. maritimum* O-AGC Type 3-0 treatment group, which exhibited severe necrosis of the kidney epithelial membrane (Figure 3I; note, no viable *T. maritimum* was recovered from this fish). No gross pathological signs or mortalities were observed in fish from the control groups except for minor scale loss caused from netting.

FIGURE 3 | Gross pathology of Chinook salmon *Oncorhynchus tshawytscha* experimentally challenged with *Tenacibaculum maritimum* via immersion. (A) Scale loss (2 days postinfection [DPI]). (B) Erythematous skin lesion (3 DPI). (C) Skin lesion with bacterial mat (3 DPI). (D) Skin ulcer under pectoral fin (3 DPI). (E) Tail necrosis and ulcer development towards caudal peduncle (3 DPI). (F) Fin necrosis and abdominal ulcer development (3 DPI). (G) Skin ulcer (8 DPI). (H) Pelvic fin and ventral haemorrhaging (2 DPI). (I) Necrotising anterior kidney epithelial membrane (8 DPI). (J) Yellow plaque on gill filament (2 DPI). (K) Eroded cleithrum bone (8 DPI). (L) Gill erosion (2 DPI).

3.2.2 | *Tenacibaculum dicentrarchi* Challenge

Tenacibaculum dicentrarchi challenged fish started showing increased opercular activity and developed pale white spots on their skin at 3 h post-infection. At 1 DPI, the first mortality was recorded, and individuals developed skin lesions. Common areas of ulceration were under the pectoral fin and the caudal peduncle. Fish exhibited deeper and larger epidermal ulcers, with the musculature exposed, compared with those of the *T. maritimum* challenge, which showed more extensive scale loss and less ulceration. A range of pathologies were observed, including deep circumscribed ulcers on the abdomen, erythematous skin lesions, mouth rot, spreading skin ulcers with bacterial mats and ulcerated caudal peduncles (Figure 4). Cumulative survival at 11 DPI was 72% ± 9.2% (Figure 2C), with 51% ± 11% of the challenged population (23% of survivors and 28% of mortalities) exhibiting skin abnormalities as shown in Figure 4. No signs of disease and no mortalities were observed in the control population.

3.3 | Pathogen Identification and Fulfilment of Koch's Postulates

3.3.1 | Bacteriology

Viable *Tenacibaculum* counts performed on the RAS tank water showed a daily reduction of bacterial load in the experimental tanks despite the disease progression in fish (Figure 5A). *T. maritimum* strains (Type 3-0: CCCM20/006; Type 2-1: CCCM20/102; and Type 3-2: CCCM20/133) and for *T. dicentrarchi* strain

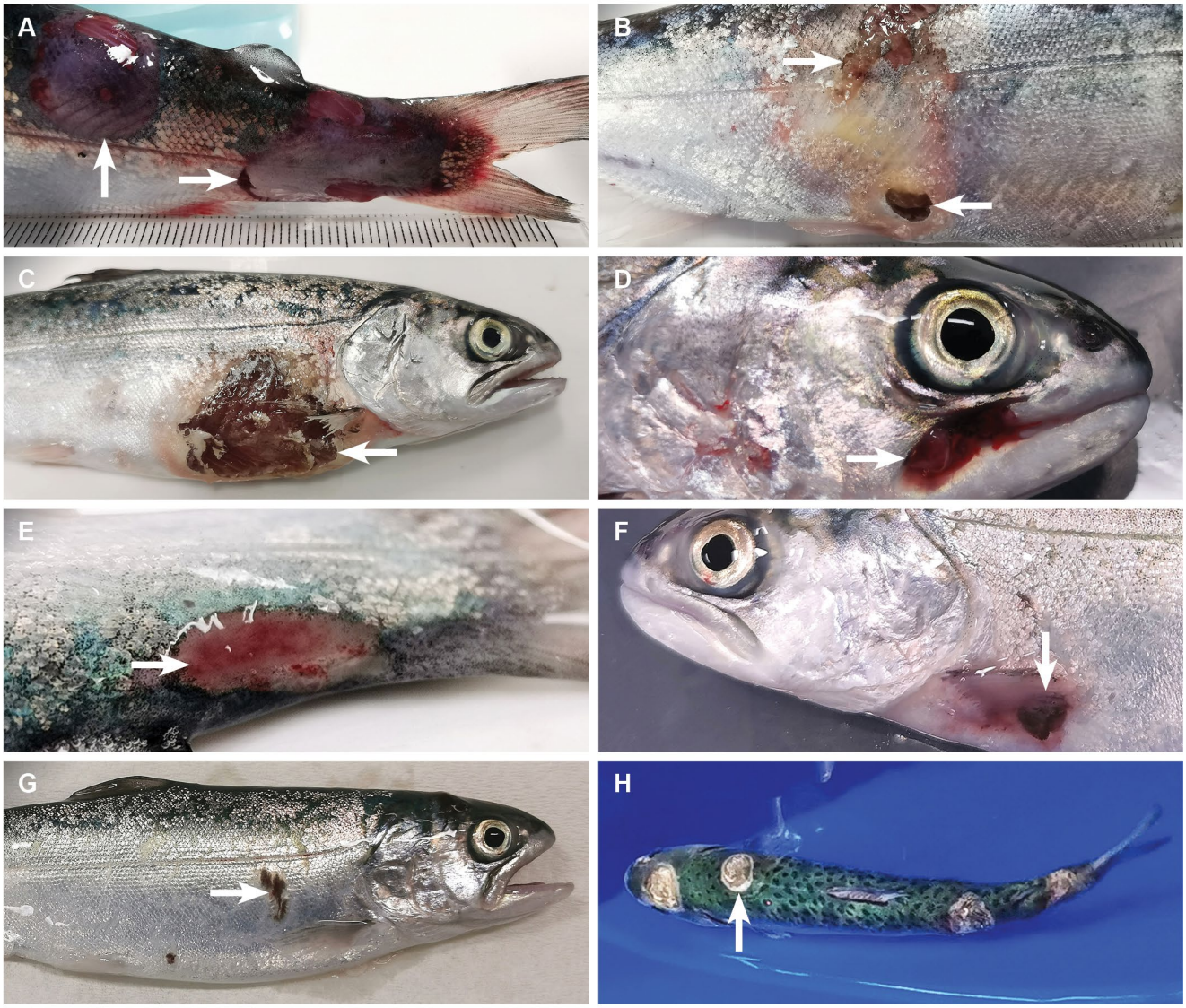

FIGURE 4 | Gross pathology of Chinook salmon (*Oncorhynchus tshawytscha*) experimentally challenged with *Tenacibaculum dicentrarchi* via immersion. (A, E) Epidermal ulceration on the caudal peduncle at 2 DPI. (B) Ulcers developing above the anal fin and expanding to the lateral line with yellow bacterial mat formation (6 DPI). (C, 9 DPI and F, 11 DPI) Moribund fish exhibiting severe inflammation and ulceration under the pectoral fin. (D) Mouth rot (11 DPI). (G) Skin lesion with intact dermis (11 DPI). (H) Moribund fish prior to removal from tank with multiple circumscribed ulcer patches (2 DPI).

(CCCM21/136) were reisolated from affected fish in the respective challenges. Bacteria were recovered in culture from overtly infected sites including the mouth, skin and gills. Recovered *T. maritimum* strains were confirmed to be the respective O-AGC type (results not shown). *T. maritimum* and *T. dicentrarchi* were not isolated from the anterior kidney in the challenged population (Figures 5B and 6C) nor from any tissue of the control population.

3.3.2 | ddPCR Analysis of *T. maritimum* and *T. dicentrarchi* Detection in Various Tissues From Infected Fish

Samples from control fish in all experimental challenge trials tested negative for *T. maritimum* and *T. dicentrarchi* by ddPCR assay. In fish challenged with *T. maritimum* (O-AGC Type 3-0) and *T. dicentrarchi*, DNA from the pathogens was detected in the

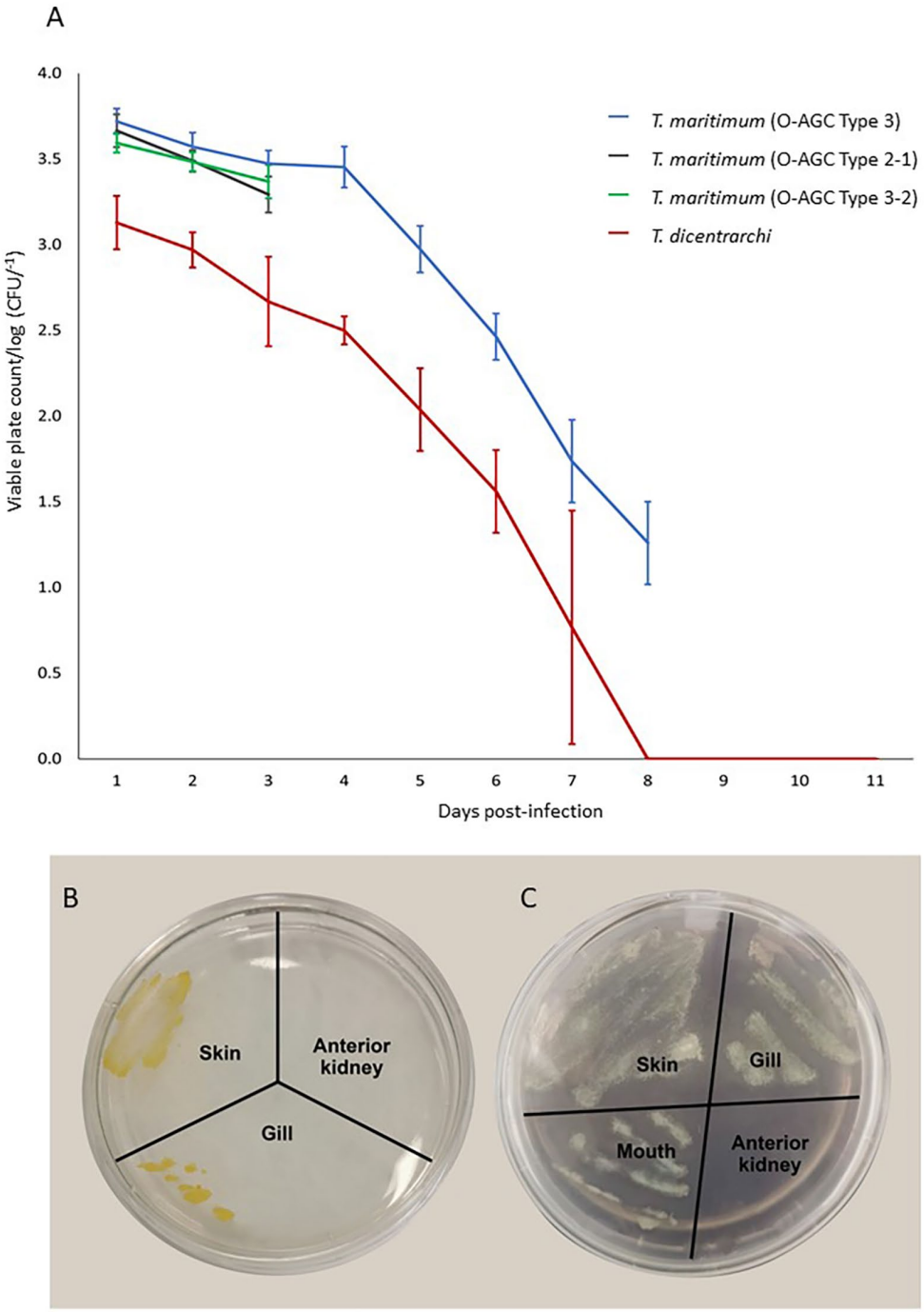

FIGURE 5 | (A) Daily average reduction of viable *Tenacibaculum* CFU in RAS tanks over the entire experimental period for each strain. *Tenacibaculum maritimum* strain O-AGC Type 2-1 and Type 3-2 experiments were ended on Day 3. (B) *Tenacibaculum dicentrarchi* and (C) *T. maritimum* isolated from infected fish skin, gills and mouth. The media showed no growth for the sample inoculated from the anterior kidney of the same individual infected fish.

skin, gills and anterior kidneys (Table S1). For fish challenged with *T. maritimum*, the highest median gene copy numbers (=34 copies/μL) were skin samples from moribund and dead fish. Highest outliers of ≥75 copies/μL were found in gill samples from moribund and dead fish. For fish challenged with *T. dicentrarchi*, copy numbers were generally lower for all tissues with the highest outliers in gill samples from moribund fish (64 copies/μL) and skin samples from moribund fish (>60 copies/μL).

Significant differences in *T. maritimum* detection were observed between the subcategories of the considered health status factors (survivors, moribund and mortality) and tissue types (Scheirer–Ray–Hare, $p<0.001$; Figure 6). For the *T. dicentrarchi* challenge, the combined factors of fish health status and tissue types were significant ($p<0.03$). Bonferroni corrected pairwise Wilcoxon rank-sum tests showed that gill and skin tissue had significantly higher *T. maritimum* loads compared with anterior kidney ($p<0.001$), and for mortality and moribund fish compared with survivors ($p<0.01$). For the *T. dicentrarchi* challenge, the pathogen load as indicated by ddPCR analyses was significantly higher in mortalities compared to survivors ($p=0.016$). However, no significant differences were observed among tissue types ($p>0.05$).

Bacterial detection between external tissue (skin and gill) versus anterior kidney by ddPCR showed significant positive correlations for *T. maritimum* (copies/mL (sqrt), p value = 0.0066). In contrast, for *T. dicentrarchi* there was no significant correlation between the pathogen load of the internal and external organs (Figure 7).

3.3.3 | Histopathology

Histopathological examination of representative samples of infected and uninfected Chinook salmon skin showed distinct differences in the epidermal integrity. The scales and overlying epidermis were absent or were severely damaged in salmon exposed to both *Tenacibaculum* spp. (Figure 8B–F). Fish infected with *T. maritimum* showed increased lymphocytic infiltration and epidermal erosion; however, bacterial cells were not present in the examined area (Figure 8B,C). The skin of fish exposed to *T. dicentrarchi* showed clusters of filamentous rods on the surface of the dermis (Figure 8D), and penetrating the dermis (Figure 8E) and underlying skeletal muscle (Figure 8F). One individual also showed extensive vacuolisation of the skeletal muscle (Figure 8D), along with increased lymphocytic infiltration.

The gills of Chinook salmon showed a wide range of pathologies following exposure to *Tenacibaculum* spp. (Figure 9). Smolt exposed to *T. maritimum* and *T. dicentrarchi* displayed widespread, mild to severe lamellar hyperplasia, especially towards the base of the gills (Figure 9C,D). There was local to widespread epithelial lifting of the secondary lamellae (Figure 9D) and local to widespread and occasionally severe degeneration of the secondary lamellae (Figure 9C,E,F). Thickening and adhesion of the secondary lamellae (lamellar fusion) were also observed, as were the presence of apoptotic erythrocytes within the secondary lamellae.

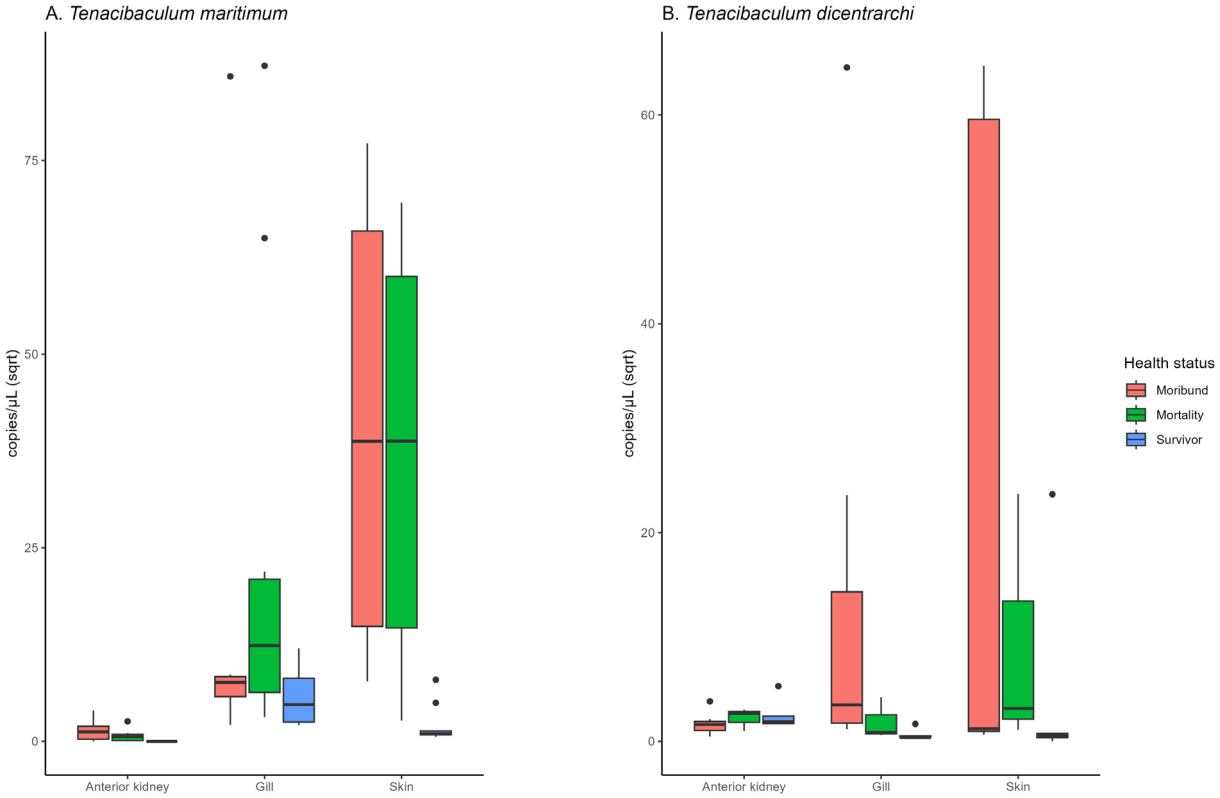

FIGURE 6 | Box plot showing the abundance of (A) *Tenacibaculum maritimum* O-AGC Type 3 and (B) *Tenacibaculum dicentrarchi* (copies/μL (sqrt)) in challenged fish from three categories (moribund, mortality and survivors) and for three tissues (anterior kidney, gills and skin). (A) Significant difference between the anterior kidney and either gill or skin (Bonferroni corrected pairwise Wilcoxon rank-sum tests; $p<0.001$). Significant difference between survivors and either moribund or mortality fish (stats test; $p<0.01$). (B) Significant difference between survivors and mortality (stats test; $p=0.016$). Black dots = outliers; Sqrt = square root transformed.

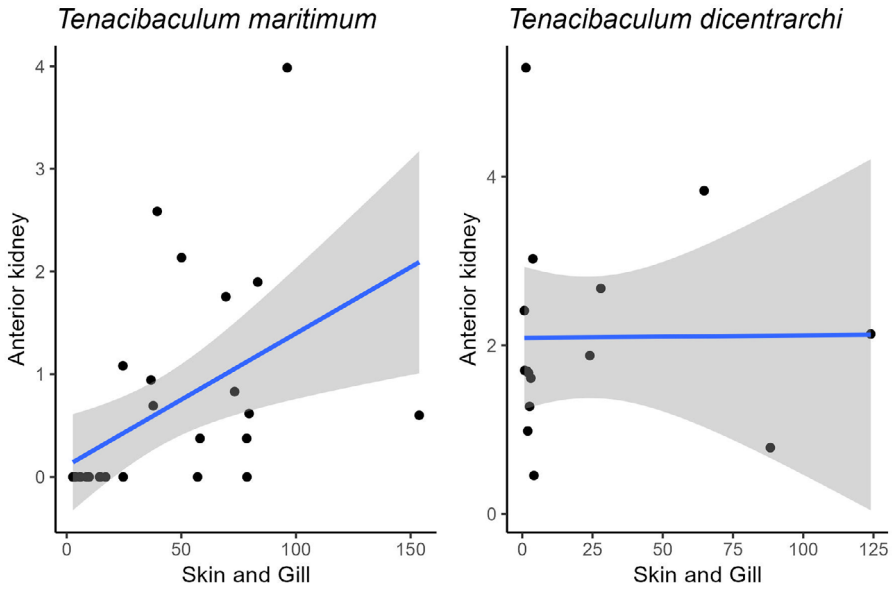

FIGURE 7 | Linear regression of square root–transformed pathogen loads (copies/mL) between anterior kidney and external tissue (skin and gill) for *Tenacibaculum maritimum* (correlation coefficient 0.49) and *Tenacibaculum dicentrarchi* (correlation coefficient 0.01), respectively.

3.4 | Viability of *T. maritimum* in External and Internal Organs of Affected Fish

Field study of fish exhibiting gross signs of tenacibaculosis from marine farms showed *T. maritimum* growth by culture media from the surface of skin, eroded mouth, gills and fins in 68% (28 out of 41 sampled; Table 2) of the population sampled. No culturable *T. maritimum* was isolated from any of the anterior kidney samples. *T. maritimum* DNA was detected in 9.7% (4 out of 41; Table 2) of infected fish, although the kidney tissue appeared to be grossly normal with no culturable *T. maritimum*.

4 | Discussion

Chinook salmon (*O. tshawytscha*) in Aotearoa New Zealand are susceptible to tenacibaculosis caused by the pathogens *T. maritimum* and *T. dicentrarchi*. Disease outbreaks in Chinook salmon associated with skin infections were first reported in 2012 in sea pens located in the Marlborough Sounds and have been consistently reported thereafter (Brosnahan et al. 2019; Kumanan et al. 2022; Ministry for Primary Industry 2017; Norman et al. 2013). Assigning disease causation and confirming whether a microorganism is an infectious agent or commensal microbiota, along with co-detection of NZ-RLOs, remains a challenge for diagnostic microbiologists, specifically for topical diseases (Brosnahan et al. 2019; Guardabassi et al. 2017; Hutson et al. 2023; Lee et al. 2023). This is the first study to provide evidence demonstrating the host–pathogen interaction between Chinook salmon and *Tenacibaculum* species. This study fulfils Koch's postulates for three *T. maritimum* strains, belonging to the O-AGC types (Type 3-0: CCCM20/006; Type 2-1: CCCM20/102; and Type 3-2: CCCM20/133), and for a single *T. dicentrarchi* strain (CCCM21/136), all of Aotearoa New Zealand origin, as pathogens of Chinook salmon. This was demonstrated through a chronological approach: pathogen association and isolation, the cultured pathogen causes disease when introduced into a healthy organism (Evans 2022; Koch 1884) and the counterfactual (Evans 2022) that establishes causal evidence of *Tenacibaculum* species as a pathogen of tenacibaculosis in Chinook salmon. We confirmed that the three strains of *T. maritimum* and *T. dicentrarchi* isolated from Chinook salmon disease outbreaks in the Marlborough Sounds were shown to induce skin disease in naïve, healthy fish in this study. Morbidity and mortality from in vivo exposures paired with clinical disease observed on farms demonstrate that Chinook salmon farmed in Aotearoa New Zealand are susceptible to tenacibaculosis. Additionally, other factors, such as environmental stressors, rising seawater temperature, water quality, and husbandry practices, could exacerbate this disease infection dynamics and contribute to the severity of outbreaks (Lane, Brosnahan, and Poulin 2022).

One of the difficulties in fulfilling Koch's postulates is establishing a functional in vivo challenge model in a controlled laboratory setting, which is essential to mimic the actual route of infection in the natural environment for a given pathogen (Bader, Nusbaum, and Shoemaker 2003). Several experimental tenacibaculosis infection methods such as bath, intraperitoneal injection, prolonged exposure up to 18 h and injury-induced infection have resulted in varying levels of success, including failure to reproduce tenacibaculosis (Bader, Nusbaum, and Shoemaker 2003; Baxa, Kawai, and Kusuda 1987; Faílde et al. 2013; Ferreira et al. 2024; Irgang and Avendaño-Herrera 2021; Jaramillo et al. 2023; Yamamoto, Kawai, and Oshima 2010). As Avendaño-Herrera, Toranzo, and Magariños (2006a, 2006b) described, the essential requirement of seawater for the growth of *T. maritimum* plays a critical role in achieving a successful culture and an in vivo challenge model. As shown in Figure S1, we obtained a homogeneous growth of *T. maritimum* when it was cultured in MSM (with natural seawater) in comparison to ZoBell's general purpose Marine Agar broth. We found that limiting the bacterial aggregation in broth is critical for achieving an accurate cell count and reproducible in vivo challenge.

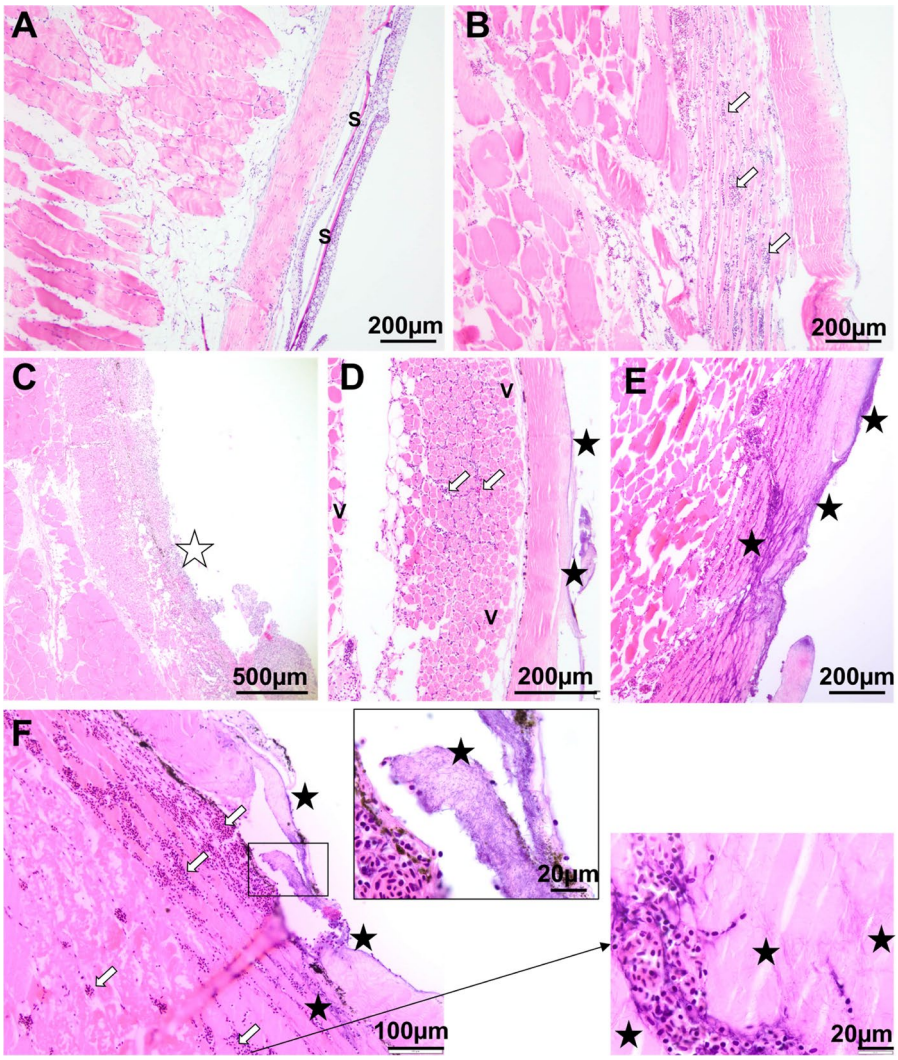

FIGURE 8 | Histopathological changes in the skin of Chinook salmon (*Oncorhynchus tshawytscha*) exposed to *Tenacibaculum* species. (A) Fish in the control group not exposed to *Tenacibaculum* spp. showing scales (S) and overlying epidermis. (B, C) Fish exposed to *Tenacibaculum maritimum*. (B) Smolt exposed to *T. maritimum* showed increased lymphocytic infiltration (white arrows) and (C) ulceration into the dermis (white star). (D–F) Fish exposed to *Tenacibaculum dicentrarchi*. (D) Vacuolisation of skeletal muscle (V) and basophilic mats of filamentous bacteria (black stars) and lymphocytic infiltration (white arrows). (E, F) Filamentous bacterial mat (black stars) underlying skeletal muscle and increased lymphocytic infiltration (white arrows).

Although many marine bacteria have been successfully manipulated using artificial seawater (He et al. 2016; Henson et al. 2016; Leifson 1963; MacLeod and Onofrey 1956), the intrinsic requirements for growth, viability and replication of *Tenacibaculum* species in natural versus artificial seawater is unknown. It is noted, however, that an in vivo trial conducted using artificial sea water to test the efficacy of an autogenous bivalent vaccine against *T. maritimum* failed to reproduce tenacibaculosis (Jaramillo et al. 2023), which suggests that natural seawater is a prerequisite for achieving infection for in vivo experimentation. Furthermore, it is evident that failing to simulate the essential characteristics of the natural environment may likely affect the microbial growth kinetics and physiology of the putative pathogen (Atolia et al. 2020; Avendaño-Herrera et al. 2006c; Medina et al. 2017; Patin et al. 2018; Stewart 2012). Immersion replicates the natural route of a non-vector–borne disease, whereby the pathogen must penetrate the host's first line of defence, the mucus barrier (He et al. 2021; McBeath et al. 2015).

We conducted the experimental infection using a 100% natural seawater bath immersion as our challenge model and were able to achieve a successful induction of tenacibaculosis in naïve Chinook salmon. Fish exposed to pathogens for a short time (1 h) at 60 kg/m^3 densities, reflecting typical industry husbandry practices during smolt transfer and grading, was sufficient for the pathogen concentration used in the trial to establish infection. The disease subsequently progressed after the infected fish were transferred to a continuous RAS system. Although a low level of *Tenacibaculum* spp. had been introduced to the RAS tanks during fish transfers from the immersion challenge bath, the daily reduction in viable cell count of *Tenacibaculum* spp. in RAS tank water suggests that the continuous UV irradiation was reducing the background load of *Tenacibaculum* spp., either due to replication or to shedding from infected fish. Progression of tenacibaculosis despite the declining *Tenacibaculum* load in tank water confirms the ability of these pathogens to adhere to fish skin, mucus or gills and to proliferate to cause disease after a short

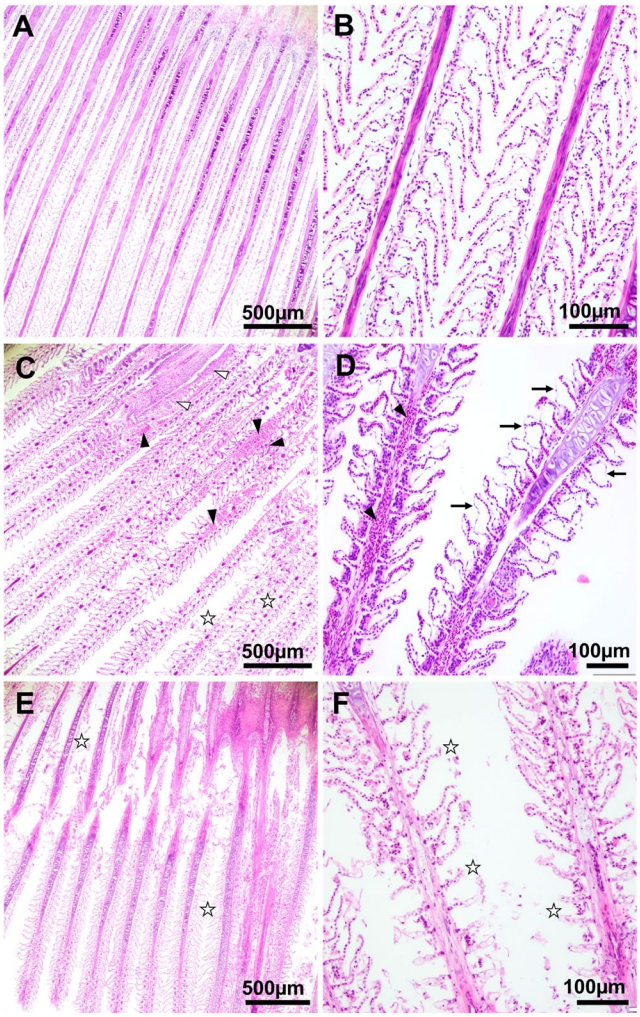

FIGURE 9 | Histopathological changes observed in the gills of Chinook salmon (*Oncorhynchus tshawytscha*) exposed to *Tenacibaculum* spp. (A, B) Normal gills of Chinook salmon not exposed to *Tenacibaculum* spp. (C) Gill filaments of Chinook salmon exposed to *Tenacibaculum maritimum* showing primary lamellar hyperplasia (black triangles), complete degeneration of the secondary lamellae (white stars) and extensive lymphocytic infiltration (white triangles). (D) Gill showing further hyperplasia (black triangles) and epithelial lifting and necrosis (black arrow). (E, F) Extensive degeneration of the secondary lamellae (white stars) shown in salmon exposed to *Tenacibaculum dicentrarchi*.

period of exposure (Echeverría-Bugueño et al. 2023; Mabrok et al. 2022).

The pathologies observed in our experimental infection of *T. maritimum* and *T. dicentrarchi* are consistent with most clinical symptoms seen in Chinook salmon summer mortality in Aotearoa New Zealand (Fischer and Appleby 2015; Johnston et al. 2020; Kumanan et al. 2022) and tenacibaculosis observed in other salmonids (Avendaño-Herrera et al. 2020; Boerlage et al. 2020; Frisch et al. 2018; Klakegg et al. 2019; Nowlan et al. 2021; Olsen et al. 2011; Småge et al. 2017; Spilsberg et al. 2022; Valdes et al. 2021; van Gelderen, Carson, and Nowak 2011). A compelling difference in the degree of skin and cartilage necrosis was seen between *T. maritimum* and *T. dicentrarchi* infection in this study. Fish infected with *T. maritimum* presented spreading but shallow skin necrosis, whereas *T. dicentrarchi* infection caused deeper ulcers extending into the musculature, along with intensive cranial and caudal peduncle cartilage necrosis. Macroscopic bacterial mats that establish on the epidermal surfaces are commonly observed for *T. maritimum* (e.g., Figure 3C). In contrast, infection caused by *T. dicentrarchi* did not exhibit this mat. Instead, they are characterised by yellow pigmentation around the edges of the ulcers and often accompanied by scale loss as shown in Figure 4B,C. The reisolation of the respective species from patches of bacterial mat and epidermal abnormalities (i.e., skin ulcer, fin necrosis and mouth rot) indicates the proliferation of the pathogens, their invasion of the mucus-epidermal layer and their ability to evade mucosal immunity (Echeverría-Bugueño et al. 2023; Escribano et al. 2020; Mabrok et al. 2022, 2016).

We noted a relationship between fish size and mortality, whereby there were survivors among the large fish challenged with *T. maritimum* O-AGC Type 3-0, but there were no survivors among the smaller fish challenged with *T. maritimum* strain O-AGC Type 2-1 and Type 3-2. The mean weight of fish challenged with O-AGC Type 3-0 was 223 ± 4.5 g, whereas for the other two serotypes, the mean weight was 120 ± 1.2 g, which suggests either that smaller fish are more susceptible to tenacibaculosis or that there is inter-strain variation in virulence. Despite the different survival rates, all three *T. maritimum* strains used in this study were able to cause disease in Chinook salmon.

Bacterial colonisation was evident grossly in the skin and gills of both *T. maritimum* and *T. dicentrarchi* infected fish; however, only the skin of fish infected with *T. dicentrarchi* showed the presence of bacterial cells in histopathological analysis. Histologically, fish infected with *T. dicentrarchi* and *T. maritimum* exhibited a range of gill pathologies including lamellar hyperplasia and fusion, which has been observed in Atlantic salmon with amoebic gill disease co-infected with *T. dicentrarchi* (see Slinger, Adams, and Wynne 2020). Despite the histologically evident gill pathologies in fish infected with *T. maritimum* and *T. dicentrarchi*, the absence of bacterial cells in histology sections of gills and *T. maritimum*–infected skin could be due to the disrupted or lost epithelial matrix during histological sample processing. Alternatively, the use of bacterial broth containing both bacterial cells and extracellular products (ECPs) secreted by the respective bacteria may have also caused hydrolysis of epithelial cells, as seen in other diseases caused by fish pathogens such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, *Photobacterium damselae* ssp. *damselae*, *P. xamselae* ssp. *piscicida*, *Streptococcus iniae* and *Y. ruckeri* (see Baiano and Barnes 2009; Ellis, Hastings, and Munro 1981; Fouz et al. 1993; Magariños et al. 1992; Mekasha and Linke 2021; V. E. Ostland et al. 2000; Pridgeon et al. 2013; Romalde and Toranzo 1993; Sarkar et al. 2021). ECPs of *T. maritimum* have demonstrated toxicity in whole animal challenge and cell lines (Michnik et al. 2024; van Gelderen, Carson, and Nowak 2009).

Bacterial pathogenesis plays a key role in determining the mechanism and development of an infection (Biondo 2022) and this understanding is crucial for the development of a viable

disease prevention option (e.g., vaccines) (Delany, Rappuoli, and Seib 2013; Mabrok et al. 2022). There are a few studies reporting that *Tenacibaculum* spp. (primarily *T. maritimum*) cause systemic infection and have been detected in internal organs of Atlantic salmon and turbot (*Psetta maxima*) (see Faílde et al. 2013; Frisch et al. 2018). However, tenacibaculosis is a topical skin disease that causes extensive damage to fish skin and cartilage (Mabrok et al. 2022) and its ability to cause septicaemia or internal infection is unclear (Lopez et al. 2022b; Småge et al. 2018).

In Chinook salmon, we found *Tenacibaculum* species infect the skin, fins and cartilage tissue; no substantial gross pathology was observed in the internal organs, such as spleen, swim bladder, liver and kidney (one exception where a *T. maritimum* challenged survivor exhibited necrotising kidney epithelial membrane). In our study, viable *Tenacibaculum* species were not re-isolated from the anterior kidney of clinically affected farmed fish or experimentally challenged Chinook salmon. Although the average seawater osmolality is approximately 1000 mOsm/kg (Dmitrieva et al. 2006; Greenwell, Sherrill, and Clayton 2003) and fish body fluid osmolality ranges from 280 to 360 mOsm/kg (Fridman 2020), the potential for *T. maritimum* to infect and proliferate in Chinook salmon internal organs is low due to its obligate requirement for seawater (Suzuki et al. 2001). However, further studies are needed as it has been observed in other fish species (Mabrok et al. 2022). Additionally, in our study, we sampled moribund fish or those that had died very recently to minimise the risk of post-mortem bacterial proliferation, which may explain why live bacteria were not detected in internal organs. The significantly low level of *Tenacibaculum* spp. detected in the anterior kidney of clinically affected farmed fish and experimentally challenged fish using ddPCR (Table 2, Figure 6) suggests that non-viable cells or nucleic acids of this pathogen had gained entry to internal organ via haematogenous spread from the site of infection from the skin (Assefa and Abunna 2018; Li et al. 2017). Four out of the 41 fish sampled from the farms were tested positive for *T. maritimum* by ddPCR (Table 2). However, for two of these fish, their skin tissue tested negative by both PCR and culture methods. This could be due to the residual DNA from vaccines given to the farmed fish, which might cause false-positive ddPCR results kidney tissue without indicating systemic infection.

The positive correlation noted in bacterial load between external tissue (skin and gill) and anterior kidney in *T. maritimum* challenged fish further proves the likelihood of hematogenous spread of pathogens. The anterior kidney in fish is a known lymphoid organ and a site for antigen presentation; therefore, during a *Tenacibaculum* infection, phagocytic antigen-presenting cells may also be responsible for the presence of nucleic acid in this tissue (Mokhtar et al. 2023). This appears consistent with the observations of Frisch et al. (2018), who found that *T. maritimum* was detected by real-time RT-PCR in several internal organs; however, fish were dying with no gross internal abnormalities other than 'yellow plaques' on the jaw in experimentally induced mouth rot in Atlantic salmon smolts. Another challenge study conducted on *Dicentrarchus labrax* reported that no *T. maritimum* was isolated from 24 h post-infected fish, although it was initially culturable at 3 and 6 h postinfection by intraperitoneal route (Ferreira et al. 2024). Even though *T. maritimum* has caused systemic infections in other fish species (Mabrok et al. 2022), *T. maritimum* and *T. dicentrarchi* strains isolated from New Zealand Chinook salmon disease outbreak did not cause systemic infection in this study. However, the secretome produced by viable bacteria at the external infection site may still have the potential to cause systemic effects (M. Pilar Escribano, Balado, Toranzo, Lemos, & Magariños, 2023; van Gelderen, Carson, and Nowak 2009). Further investigation with a prolonged experimental duration to achieve chronic infection is needed to fully understand the role of *Tenacibaculum* species in the systemic health of Chinook salmon affected by tenacibaculosis.

In conclusion, this study has fulfilled Koch's postulates for the *T. maritimum* strains CCCM20/006, CCCM20/102 and CCCM20/133 from O-AGC Type 3-0, Type 3-2 and Type 2-1, respectively, and for *T. dicentrarchi* (CCCM21/136), which are independently causative agents of tenacibaculosis in Chinook salmon. This study also serves as a starting point for further research on Chinook salmon, where several other factors can be evaluated including co-infection with multiple *Tenacibaculum* species and challenge using lower bacterial dose to understand the role of pathogens in winter. Our natural disease challenge model could be used to explore disease-resilient breeding for more sustainable production amid increasing seawater temperatures linked to directional climate change (Bai and Plastow 2022) and would also have significant value in assessing the efficacy of prototype *Tenacibaculum* vaccines.

Author Contributions

Karthiga Kumanan: conceptualization, project administration, data curation, formal analysis, investigation, methodology, resources, validation, visualization, writing – original draft. **Jeremy Carson:** conceptualization, supervision, writing – review and editing. **Ryan B. J. Hunter:** investigation, validation, resources, writing – review and editing. **Anne Rolton:** writing – original draft, investigation, visualization. **Ulla von Ammon:** investigation, visualization, writing – review and editing. **Chaya Bandaranayake:** investigation. **Connie Angelucci:** conceptualization, writing – review and editing. **Richard N. Morrison:** writing – review and editing. **Seumas P. Walker:** resources. **Jane E. Symonds:** resources, writing – review and editing. **Kate S. Hutson:** funding acquisition, project administration, supervision, methodology, conceptualization, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.