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RESEARCH ARTICLE

Skin-Mucus Prokaryote Community of Atlantic Salmon (*Salmo salar*) in Response to Bath Challenge With *Tenacibaculum dicentrarchi*

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ABSTRACT

Fish skin mucus is continuously replaced by epidermal cells, making it a highly dynamic microenvironment and an effective barrier against waterborne pathogens. The objective of this study was to understand the effects of tenacibaculosis, caused by the bacterium *Tenacibaculum dicentrarchi*, on the skin-associated microbiome of Atlantic salmon (*Salmo salar*). We used a vector-free and waterborne infection model of *T. dicentrarchi* strain TdCh05 in Atlantic salmon smolts for 21 days. Skin swab samples were collected at 2 h and 21 days post-infection (hpi and dpi, respectively) for 16S rRNA gene amplicon sequencing using DNA or complementary DNA (cDNA) as templates. Non-metric multidimensional scaling analysis grouped the samples into distinct clusters depending on the treatment and template. Similarity-Percentage (SIMPER) analysis indicated that between ~42% and 43% of the total amplicon sequence variants (ASVs) across all samples accounted for 90% of the compositional differences among all treatments and the two templates, highlighting the contribution of *Tenacibaculum* ASVs. Comparisons (by SIMPER) between non-infected and TdCh05-challenged fish at 2 hpi indicated that *Tenacibaculum* ASVs contributed to between ~52% and 58% of the differences in compositional clustering between samples. A significant drop in skin-mucus alpha diversity in TdCh05-challenged fish was also detected, followed by alpha diversity recovery at 21 dpi. In turn, at 21 dpi, microbiome changes were related to higher interaction complexity among taxa and community instability. Furthermore, 16S cDNA-based sequencing indicated that the potential activity of the Atlantic salmon skin-associated microbiome during disease progression was primarily driven by *Tenacibaculum* spp. Further research is needed to elucidate the role of other potentially active components (e.g., *Pseudomonadales*) of the skin-associated microbiome for the onset and/or progression of tenacibaculosis.

1 | Introduction

Microbial communities living in or on animal bodies have traditionally been referred to as 'microflora'. One of the earliest studies of fish skin microflora dates to the late 1950s and examined bacteria on the skin of North Sea cod (*Gadus morhua*) (Georgala 1958). This and other related studies at the time relied on traditional culture-dependent methods, which initially led to some misconceptions, such as the notion that the composition of the skin flora was independent of the host fish species (Georgala 1958). In the same sense, the low number of bacteria per square centimetre of fish skin (i.e., 10^2 to 10^4) (de Bruijn et al. 2018) was initially associated with low diversity and dominance by a few taxa. For example, the abundance of culturable skin-associated bacteria in Atlantic salmon (*S. salar*) typically ranges from 10^2 to 10^3 bacteria per square centimetre of skin (Benhamed et al. 2014). This range of abundance does not reflect the overall skin-associated microbial richness or diversity for Atlantic salmon, which in its seawater stage is mostly represented by phylotypes belonging to Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia and Firmicutes (Lokesh and Kiron 2016; Minniti et al. 2017; Brown et al. 2021; Lorgen-Ritchie et al. 2022).

Over time, the term "microflora" evolved into "microbiota" to describe the structure and abundance of microbial communities found inside animal bodies and/or their environments (Marchesi and Ravel 2015). More recently, the term microbiome was coined to emphasise the fact that the microbiota is a source of both genetic variability and metabolites (Hoffmann et al. 2016). Although microbes often get a bad reputation, the implementation of omics tools in microbiome research (Biteen et al. 2016) has allowed this notion to change. For instance, fish microbiomes have been proposed as virtual endocrine systems capable of generating and recognising compounds that interact with the nervous, endocrine, immune and reproductive systems (Garcia-Reyero 2018; Haque et al. 2022).

The fish skin-associated microbiome consists of a microbial community with an aerobic metabolism (Leonard et al. 2014) and a taxonomic composition that depends on the surrounding environment (Carda-Diéguez et al. 2017), fish species and populations (Larsen et al. 2013; Berggren et al. 2022), diet (Chiarello et al. 2018), host-specific factors and water chemistry (Sylvain et al. 2016). The skin microbiome of Atlantic salmon can be influenced by environmental conditions (e.g., changes in water salinity and temperature) (Lokesh and Kiron 2016; Lorgen-Ritchie et al. 2023; Bell et al. 2024), microenvironmental effects through the influence of tank biofilms (Minich et al. 2020), dietary components (Landeira-Dabarca et al. 2013; Bledsoe et al. 2022), fish netting and transfer (Minniti et al. 2017) and developmental stage and conditions experienced during early life (Uren Webster et al. 2020), such as acute cold stress during late embryogenesis (Uren Webster et al. 2021). Furthermore, the transition from freshwater to seawater is known to reshape the skin-associated microbiome of Atlantic salmon (Lokesh and Kiron 2016).

Hosts may enter a state known as dysbiosis when an abrupt decrease in richness and relative abundance of key taxonomic groups belonging to commensal microbiome phyla is noticeable and persistent (Lorgen-Ritchie et al. 2022). Interactions between the host, microbiome and certain nonbacterial pathogens (e.g., viruses and ectoparasites) may promote skin dysbiosis (Reid et al. 2017; Valenzuela-Miranda et al. 2024). Skin dysbiosis could in turn lead to secondary bacterial infections by endogenous opportunistic pathogens or decreased resistance to colonisation by exogenous pathogens (e.g., those of the genera *Vibrio*, *Flavobacterium*, *Tenacibaculum* and *Pseudomonas*) in Atlantic salmon with high louse burdens (Llewellyn et al. 2017). Alternatively, Atlantic salmon can recover from skin dysbiosis without compromising immune function or growth, suggesting a high resilience of the mucosal microbiome (Lorgen-Ritchie et al. 2022). Indeed, balanced microbiomes provide an important barrier against microbial infections by outcompeting pathogens and/or stimulating the host immune system (Gomez et al. 2013). For instance, the first gnotobiotic experimental system in Atlantic salmon yolk sac fry showed that the mucus barrier is less protective in germ-free fry compared to fry colonised by bacterial communities (Gómez de la Torre Canny et al. 2023).

Tenacibaculum dicentrarchi, *Tenacibaculum finnmarkense* and *Tenacibaculum maritimum* are three marine bacterial pathogens associated with external clinical manifestations of a fish disease known as tenacibaculosis (Olsen et al. 2019). A significant dysbiosis during a natural outbreak of tenacibaculosis was primarily driven by *T. maritimum* in Atlantic salmon in western Canada, but *T. dicentrarchi* and other species of *Tenacibaculum* were also detected in low abundances (Wynne et al. 2020). In other regions, tenacibaculosis caused by *T. dicentrarchi* can be a major health threat to Atlantic salmon (Avendaño-Herrera et al. 2016; Klakegg et al. 2019) and other fish species (Piñeiro-Vidal et al. 2012; Nikouli et al. 2019; Saldarriaga-Córdoba et al. 2021). According to *in vitro* experiments, the skin mucus of healthy and diseased Atlantic salmon may be ineffective in protecting fish from *T. dicentrarchi* (Echeverría-Bugueño et al. 2023). In addition, lesions induced by primary infections in external mucosal surfaces of Atlantic salmon may lead to a complex interplay with *T. dicentrarchi* as a source of secondary infections. For example, lesions in gill tissue of Atlantic salmon with amoebic gill disease (caused by *Neoparamoeba perurans*) showed a significantly higher abundance of *T. dicentrarchi* compared to adjacent tissue without visible lesions (Slinger et al. 2020). In addition, *T. dicentrarchi* was prominently detected on skin ulcers of Atlantic salmon infected with *Piscirickettsia salmonis*, suggesting a complex dynamic of coinfection between both species and with other opportunistic pathogens, as well as a potential dysbiosis (Godoy et al. 2023).

To gain further insight into the progression of *T. dicentrarchi*-mediated tenacibaculosis in Atlantic salmon, we examined changes in the skin mucus prokaryote microbiome of infected fish at different times during experimental infection. Our aim was to determine the main effects of Atlantic salmon colonisation by *T. dicentrarchi* on the composition of the salmon skin-associated microbiome and to detect potential effects on the interactions of the major microbial components.

2 | Materials and Methods

2.1 | Overall Experimental Setup

Healthy Atlantic salmon (~170 g) specimens were obtained from a freshwater fish farm, with no history of diseases, in

the Los Lagos Region of Chile. Before transport (~1320 km), the fish were certified as pathogen-free, including from infectious pancreatic necrosis virus, *Flavobacterium psychrophilum*, *Yersinia ruckeri*, infectious salmon anaemia virus, *P. salmonis*, *Renibacterium salmoninarum*, *T. maritimum*, *T. dicentrarchi* and other *Tenacibaculum* species. This status was given in accordance with the regulations established by the Chilean National Fisheries and Aquaculture Service. Diagnostic procedures included standard microscopic and bacteriological analyses, as well as PCR testing, all conducted by an accredited private laboratory. All experimental procedures and animal handling adhered to ethical standards set by CONICYT and the Universidad Andrés Bello Ethics Committee under approval accreditation 005/2019. The experiments were conducted at the Experimental Unit for Challenge Trials at CIMARQ, Universidad Andrés Bello, Quintay, Valparaíso, Chile. Fish were acclimated in two 600 L continuous-flow seawater tanks for 2 weeks at 16°C ± 1°C with aerated seawater (36‰) before the *T. dicentrarchi* challenge. They were fed commercial food pellets at 1.5% body weight daily. The photoperiod consisted of 12:12 light: dark.

The *T. dicentrarchi* strain TdCh05, isolated from an outbreak of tenacibaculosis in Atlantic salmon in 2014 and confirmed to have pathogenic potential in different salmonid species (Avendaño-Herrera et al. 2016), was chosen for this study. This strain has been studied at the genomic level (Saldarriaga-Córdoba et al. 2021), and research further exists on virulence mechanisms associated with iron (Avendaño-Herrera et al. 2023, 2024) and outer membrane vesicles (Echeverría-Bugueño and Avendaño-Herrera 2024). Before experimentation, strain TdCh05 was verified as *T. dicentrarchi* using a previously described PCR protocol (Avendaño-Herrera et al. 2018), and culture purity was assessed by microscopic observation.

The strain was routinely grown on *Flexibacter maritimum* medium (FMM) (Pazos et al. 1996) in agar plates or broth and incubated at 18°C for 48–72 h. For the bath challenge, FMM broth cultures were prepared and incubated under the same conditions as for routine growth, but with agitation (100 rpm). The concentration of *T. dicentrarchi* was determined by the number of cells per millilitre (cells/mL) under a light microscope at 1000× magnification. In addition, the number of colony-forming units per millilitre (CFUs/mL) was determined using serial dilutions of the inoculum and subsequent plating on FMM agar plates.

A total of 40 fish were used in this challenge study. The pre-challenge state of fish was determined from fish kept in 600 L continuous-flow seawater tanks. Virulence tests were performed by immersing 14 fish in 40 L plastic tanks containing seawater inoculated for 2 h with FMM broth cultures to obtain at a final concentration of 5×10^6 TdCh05 cells/mL, with constant aeration. Another group of 14 fish was exposed to an FMM bath (i.e., a mixture of sterile FMM broth and seawater in the same proportion as used in the virulence tests) in 40 L plastic tanks for 2 h with constant aeration. A third group of fish ($n = 14$) was used as a control for monitoring. Afterward, all fish from each group were transferred to the recirculating aquaculture system room and distributed in subgroups of seven fish into 100 L plastic tanks (80 L working volume) at ~15 kg/m^3. Fish feeding was daily, and faeces were removed every other day. The recirculating aquaculture system was operated between 16°C and 17°C, at a salinity between 30% and 32‰, and with constant aeration over a period of 21 days, during which time each group was observed daily for clinical signs of tenacibaculosis. Dead fish were removed from the tank daily (these fish were not included in the sample analysis) to assess whether the inoculated bacterium was responsible for the mortality. This included streaking FMM agar with skin lesions and/or tissue samples from the spleen, liver, kidney and brain tissues. The suspected colonies were identified through phenotypic tests, and PCR analysis was performed on the isolates and tissue samples to confirm that the fish mortalities observed in the experimental tanks were caused by *T. dicentrarchi* TdCh05.

2.2 | Sample Collection and DNA and RNA Extractions

A total of 30 samples (see description labels in Figure 1) were collected by skin swabbing (GenoTube swabs; Applied Biosystems) from seawater-stage Atlantic salmon for skin-associated microbiome analysis from DNA and cDNA templates. All selected Atlantic salmon specimens did not present any detectable clinical signs of tenacibaculosis. Before skin swabbing, fish were sedated with BZ-20 at 30 mg L^{-1} in seawater (Veterquímica). Briefly, skin sampling was performed by moving a swab along the lateral line of the fish, rotating it over the surface of the fish from the operculum to the tail. Triplicate swab samples (each from a different randomly selected fish) were collected from (1) the pre-challenge state to determine the skin-associated microbiome of fish not exposed to *T. dicentrarchi* TdCh05 and (2) non-infected fish in the FMM bath to detect any enrichment of natural *Tenacibaculum* and its influence on initial changes in microbiome composition. Additionally, triplicate swab samples were collected from (3) TdCh05-challenged fish at 2 h post-infection (hpi) and (4) 21 days post-infection (dpi), as well as from (5) control fish at 21 dpi. The selection of sampling times was based on previous infection studies that demonstrate the fish mortality curve primarily occurs within the first 7 dpi (Avendaño-Herrera et al. 2016) and the results from biofilm formation studies (Levipan et al. 2019). Afterward, fish were returned to their respective recirculating aquaculture system tanks for recovery. Swab samples were stored at −80°C until DNA and RNA extractions for subsequent skin-associated microbiome analysis.

DNA was extracted from swabs using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) following the manufacturer's instructions with an additional disruption step. Swab tips were cut with sterile tweezers and scissors and placed into PowerSoil lysing bead tubes for cell disruption on a TissueLyser II (Qiagen, Hilden, Germany). The TissueLyser was set for 2×1 min at a frequency of 30 Hz with a 1-min pause between sessions. Similarly, RNA was extracted from swabs using the Ambion RNA Extraction Kit (AM1560, Ambion, Austin, TX, USA) and the manufacturer's guidelines. The same mechanical disruption conditions and 200 μm diameter zirconium beads (Low Binding Zirconium Beads, OPS Diagnostics, Lebanon, NJ) were used on a TissueLyser II. The concentration of DNA and RNA extracts was determined on a Qubit 4 fluorometer (Thermo Fisher Scientific, MA, USA) using the Qubit dsDNA BR and RNA HS

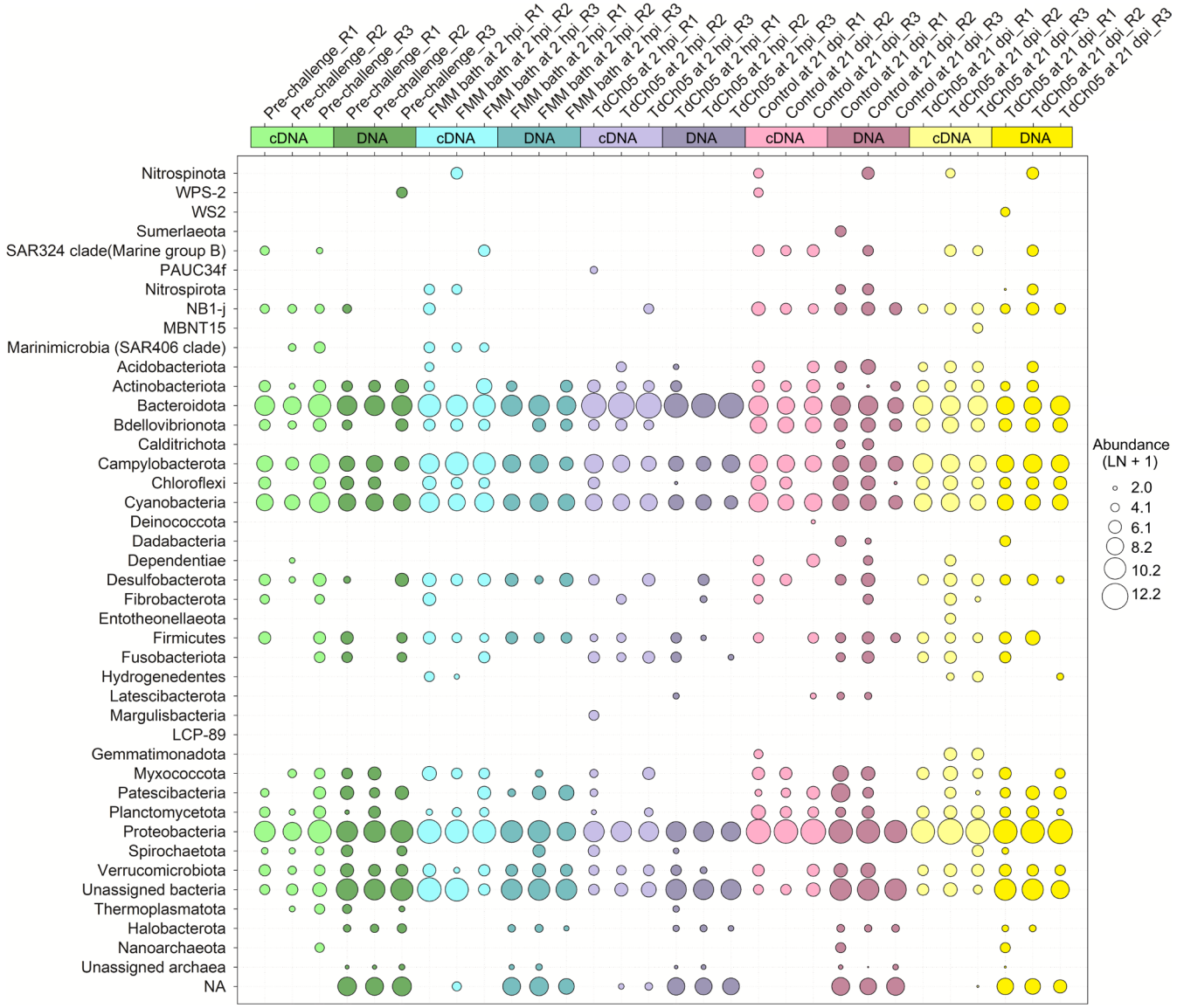

FIGURE 1 | Bubble chart of the abundance of ASVs grouped at the phylum level for the Atlantic salmon skin-associated microbiome. Bubble size represents abundance as the LN + 1 transformed number of read counts. The colour key on the top x-axis shows the type of template (cDNA or DNA) used for 16S rRNA amplicon sequencing from swab samples collected in triplicate (R1 to R3) from each treatment. Prokaryote phyla, which were not assigned (NA) sequences, and unassigned bacterial and archaeal sequences are shown on the left y-axis.

assays, respectively (Thermo Fisher Scientific). The integrity of RNA extracts was determined using the Qubit RNA IQ Assay Kit per the manufacturer's instructions (Thermo Fisher Scientific). DNA and RNA extracts were stored at −20°C and −80°C, respectively, until further analysis.

2.3 | Synthesis of Complementary cDNA

Trace amounts of DNA in RNA preparations were removed with the TURBO DNA Free Kit (Applied 500 Biosystems, Austin, TX, USA). DNA-free RNA extracts were used for the synthesis of cDNA using the ImProm-II Reverse Transcription System in accordance with the manufacturer's instructions (Promega, WI, USA). Reverse transcription reactions were performed using 20 ng of DNase-treated RNA and the random primer set provided by the manufacturer. The resulting cDNAs were quantified using the Qubit ssDNA Assay Kit (Thermo Fisher Scientific) and stored at −20°C for subsequent 16S rRNA gene-amplicon sequencing.

2.4 | 16S DNA- and 16S cDNA-Based Amplicon Sequencing and Taxonomic Assignment

Amplicon-based libraries targeting the V4 region of 16S rRNA genes were constructed by Genoma Mayor SpA (http://www.genomamayor.com/) from DNA and cDNA preparations. The primer pair 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 806RB (5′-GGACTACNVGGGTWTCTAAT) was modified with specific Illumina adapters and barcodes following standard protocols (http://www.earthmicrobiome.org/protocols-and-standards/16s/). PCR reactions used the 2× KAPA HiFi HotStart Ready Mix (KAPA Biosystems, MA, USA), and amplicons were purified with AMPure XP (Beckman Coulter, CA, USA). Quantification

was performed using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, OR, USA) on a HOEFER DQ300 fluorometer (Hoefer Inc., MA, USA). The size of the PCR product was verified by using the DNF-900 Kit on a Fragment Bioanalyzer (Advanced Analytical Technologies Inc., IA, USA). DNA pooling and sequencing preparation used standard protocols in accordance with the Illumina Denature and Dilute Libraries Guide. Sequencing was performed on the Illumina HiSeq 250PE platform using around 100,000 reads per library. The raw sequence data are downloadable from the NCBI SRA database under the BioProject ID PRJNA1208091.

Low-quality sequences were filtered, and raw data were trimmed using the DADA2 v1.26.0 R package (Callahan et al. 2016). The quality-processed paired-end reads (USEARCH method) (Edgar and Flyvbjerg 2015) were used to generate Amplicon Sequence Variants (ASVs) using the DADA2 pipeline (Callahan et al. 2016). Taxonomic identification of ASVs was performed using the Naive Bayesian Classifier algorithm trained for V4 sequences of 16S rRNA genes (Wang et al. 2007). Data visualisation employed the phyloseq package from the R package (McMurdie and Holmes 2013).

2.5 | Statistical Analysis

Changes in Atlantic salmon skin-associated microbiome composition (at the ASV level) were detected by non-metric multidimensional scaling (NMDS) ordination using the Bray-Curtis distance to detect clusters of samples separated by treatment and template. Permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis distance with 9999 permutations (Anderson 2001) was performed to evaluate significant changes in composition between groups resulting from NMDS ordinations. Similarity-Percentage (SIMPER) analyses were performed to identify ASVs that contributed most to the differences between treatments and between templates. In addition, a two-way analysis of variance (ANOVA) was performed to assess significant changes in alpha diversity of the salmon skin-associated microbiome. This tested the effects of the treatment and template factors on the Shannon diversity index. Post hoc analysis using Tukey's Honest Significant Difference test detected significant differences between factor levels. All these analyses were performed using the vegan package (Oksanen et al. 2015) in R v4.3.0 (R Core Team 2023).

Undirected interaction networks based on ASV abundance matrices were constructed from 16S cDNA- and 16S DNA-based amplicon sequencing data and grouped at the order level (except for unassigned sequences at this level, which were grouped at the class level or higher) for two clusters of samples. The first group consisted of samples collected from the pre-challenge state and FMM bath (i.e., two initial treatments of unchallenged fish), and the second one was composed of samples from TdCh05-challenged fish at 21 dpi. Spearman rank correlations were performed to define microbial interactions. A given correlation (regardless of the statistical significance) was considered an interaction of interest if its Spearman's correlation coefficient was between −0.6 and −1 (for co-exclusion interactions) or between 0.6 and 1 (for co-occurrence interactions). To compare network topology, the following network properties were analysed: network size, network degree, modularity (Alcalá-Corona et al. 2021; Newman and Girvan 2004), degree assortativity (Newman 2002) and degree distribution (Ávila-Thieme et al. 2023; Dunne et al. 2008). All isolated nodes were removed from the networks. Four types of centrality measures (node properties) were determined to detect keystone taxa in the networks (Banerjee et al. 2018): total degree (Delmas et al. 2019), closeness degree (Banerjee et al. 2018; Delmas et al. 2019), betweenness degree (Banerjee et al. 2018; Delmas et al. 2019) and eigenvector (Allesina and Pascual 2009). A keystone index was calculated by considering the rank of each centrality measure. To do so, each centrality measure was separately transformed into a rank. Then, for each taxon, the rank of each centrality measure was summed and ranked. Taxa in the top 5% of the keystone index were used to generate a list of the most important members of the networks. The proportion of networks with each of these taxa was calculated for comparison purposes. All indices were calculated using igraph R (Csárdi et al. 2024), while the degree distribution was computed using the NetworkExtinction R package (Ávila-Thieme et al. 2023). All these analyses were performed in R v4.3.0 (R Core Team 2023).

3 | Results

3.1 | Fish Mortality by Bath Challenge: Virulence Assays

The virulence assay using the *T. dicentrarchi* isolate TdCh05 resulted in a cumulative mortality of 57.14% (8 out of 14 fish), with the first mortality recorded on Day 2 post-challenge and the last on Day 15 (data not shown). Initially, the three fish that died within the first 3 days exhibited external damage such as scale loss, gill damage and ulcer-like lesions on both lateral flanks. The mortalities occurring between Days 14 and 15 presented with ulcers, frayed fins, and even complete loss of the tail, leaving the caudal peduncle exposed. Additionally, lesions were observed on the snout of the fish, accompanied by haemorrhaging. These five fish also showed macroscopic lesions in internal organs, specifically liver inflammation, pallor, haemorrhaging in the pyloric caeca, and ascites (data not shown).

Microbiological sampling on FMM agar plates allowed recovery of the TdCh05 strain from all deceased fish, particularly from external lesions. Moreover, tissue samples from which *T. dicentrarchi* TdCh05 could not be cultured tested positive by PCR, confirming that the inoculated bacterium was the cause of death. In contrast, no mortality or macroscopic lesions were observed in the 14 fish bathed in FMM medium or in the control group during the 21 days post-challenge.

3.2 | Compositional Variation of the Salmon Skin-Associated Microbiome

Considering all treatments, a total of 12,301 ASVs were recovered, of which 9,418 were assigned to 39 prokaryotic phyla, and the rest remained unassigned prokaryotic ASVs (Figure 1). Bacterial ASVs were the dominant components of the salmon skin-associated microbiome, but unassigned archaea and three archaeal phyla (i.e., Nanoarchaeota, Halobacterota and Thermoplasmatota) were also detected in low abundances (Figure 1). The top 10 bacterial phyla in decreasing order of

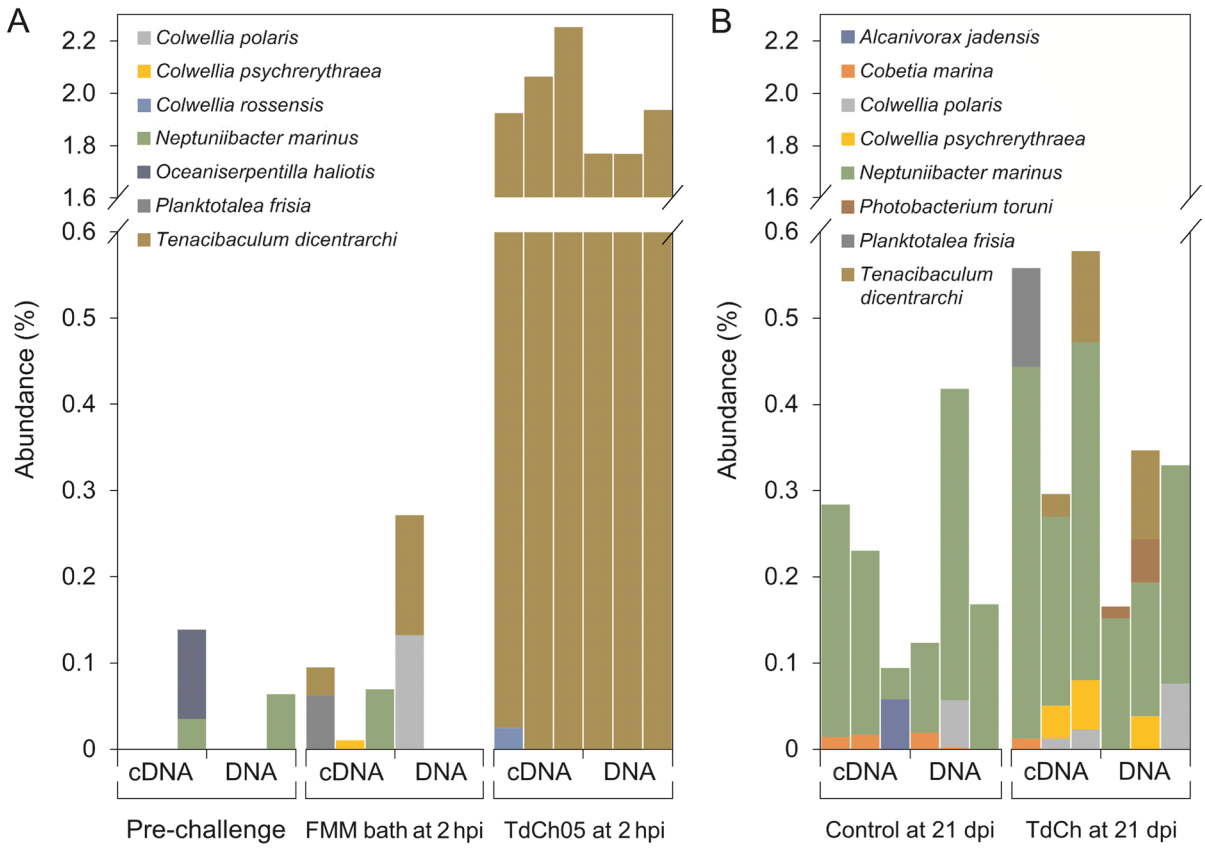

FIGURE 2 | Bar plot showing the top 10 ASVs grouped at the species level for the Atlantic salmon skin-associated microbiome. The relative abundance is shown as a percentage of the total number of reads obtained from cDNA and DNA templates. Triplicate samples were collected from fish (A) in the pre-challenge state, FMM bath and TdChD05 challenge at 2 hpi. (B) Triplicate samples were also collected from control and TdChD05-challenged fish at 21 dpi.

abundance were Proteobacteria, Bacteroidota, Campylobacterota, Cyanobacteria, Bdellovibrionota, Patescibacteria, Chloroflexi, Verrucomicrobiota, Myxococcota and Desulfobacterota (Figure 1). The top 10 bacterial genera belonged to Proteobacteria, with the exception of the fish pathogen *T. dicentrarchi* (phylum Bacteroidota), which was dominant at 2 hpi (Figure 2A) and decreased in abundance at 21 dpi (Figure 2B).

NMDS ordinations based on the changes in the skin-associated microbiome of Atlantic salmon (at the ASV level) revealed different clusters of samples that distinguished between different treatments (Figure 3A) and templates (Figure 3B). Significant differences ($p < 0.01$) in skin-associated microbiome composition for identified clusters (Figure 3) were detected by a (Tables S1–S6) PERMANOVA (Table 1). This analysis revealed no significant effect of the treatment × template interaction on taxa compositional changes (Table 1).

ANOVA analysis indicated that the factor treatment had a significant effect on changes in the alpha diversity (Shannon diversity index) of the Atlantic salmon skin-associated microbiome. The factor template and the treatment × template interaction had no significant effect on alpha diversity (Table 2). There were significant differences in alpha diversity between skin samples taken from TdCh05-challenged fish at 2 hpi and fish-skin samples collected from the remaining treatments (Figure S1).

SIMPER analysis identified the ASVs that contributed most to the detected differences between treatments and templates. The overall mean dissimilarity in skin-associated microbiome composition between all treatments (Figure 3A) and between the two templates (Figure 3B) was equal to 92.42% and 89.78%, respectively (Tables S1 and S2). Of the total number of ASVs, approximately 42% (i.e., 5,184 out 12,301 ASVs) and 43% (i.e., 5,308 out 12,301 ASVs) accounted for 90% of the compositional differences among all treatments and the two templates, respectively (Tables S1 and S2). However, only the first 370 and 439 ASVs of the SIMPER results (Tables S1 and S2) accounted for ~50% of these differences in taxa composition; these ASVs mostly belonged to the genera *Tenacibaculum, Oleispira*, *Neptuniibacter* and *Spongiibacter* (Tables S1 and S2). Particularly, *Tenacibaculum* ASVs (including *T. dicentrarchi* ASVs) represented ~28% (i.e., 102 out 370 ASVs) (Table S1) and ~23% of the ASVs (i.e., 102 out 439 ASVs) (Table S2) contributing to the aforementioned compositional-difference level. In fact, a SIMPER comparison between the pre-challenge state and TdCh05-challenged fish at 2 hpi indicated that *Tenacibaculum* ASVs alone contributed to ~58% of the differences in compositional clustering between samples (Table S3). Similarly, in a SIMPER comparison between FMM bath treatment and TdCh05-challenged fish at 2 hpi, *Tenacibaculum* ASVs along with seven *Arcobacteraceae* ASVs contributed to ~52% of the differences between samples (Table S4).

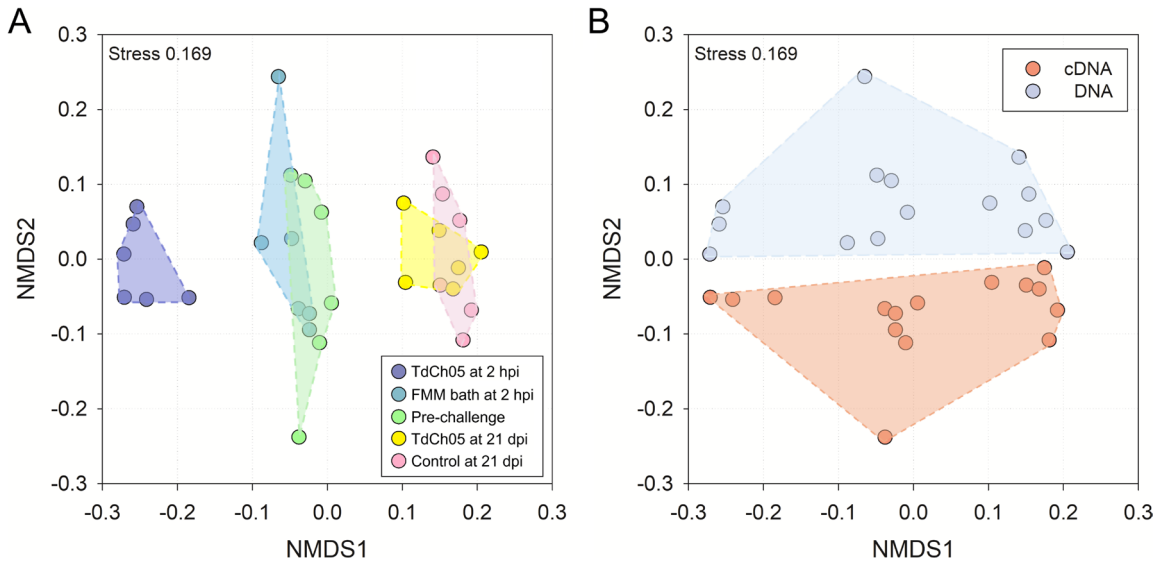

FIGURE 3 | Changes in skin-associated microbiome composition of Atlantic salmon. NMDS ordinations show differences in composition at the ASV level for triplicate samples from different treatments and nucleic acid templates. (A) Clusters of samples per treatment: Samples from TdChD05-challenged fish at 2 hpi (in purple), samples from fish in the pre-challenge state (in green) and after FMM bath (in cyan), and samples from control (in pink) and TdChD05-challenged fish at 21 dpi (in yellow). (B) Clusters of samples based on the type of template. Distinctive clustering of samples was supported by PERMANOVA analysis ($p < 0.001$). All ordinations were performed using the Bray-Curtis distance.

TABLE 1 | Results of two-way PERMANOVA testing the effects of the treatment and template on the composition of the skin-associated microbiome of Atlantic salmon.

Variable	Effect	SS	DF	MS	*F*
Skin-associated microbiome composition	Treatment	5.02	4	1.26	5.21***
	Template	0.74	1	0.74	3.05**
	Treatment × template	1.07	4	0.27	$1.11^{n.s}$
	Residual	4.82	20	0.24	
	Total	11.65	29		

Note: The level of statistical significance for each effect is indicated by asterisks ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***). *p* values were obtained after 9999 permutations.
Abbreviations: DF, degree of freedom; F, F-statistic; MS, mean square; n.s, non-significant effect; SS, sum of squares.

TABLE 2 | Two-way ANOVA testing the effects of treatment and template factors on the alpha diversity (Shannon index) of the skin-associated microbiome of Atlantic salmon.

Variable	Effect	SS	DF	MS	*F*
Shannon diversity index	Treatment	4.37	4	1.09	8.87***
	Template	0.37	1	0.37	$3.00^{n.s}$
	Treatment × template	1.09	4	0.27	$2.22^{n.s}$
	Within	2.47	20	0.12	
	Total	8.30	29		

Note: The level of statistical significance for each effect is denoted by asterisks ($p < 0.001$ = ***).
Abbreviations: DF, degrees of freedom; F, F-statistic; MS, mean square; n.s, non-significant effect; SS, sum of squares.

3.3 | Network Analysis of Microbial Taxa

The DNA-based network size did not vary between treatments (i.e., between the pre-challenge and FMM bath states and the challenge state at 21 dpi), except by co-exclusion networks that increased in size after 21 dpi in 30 taxa. In contrast, the cDNA-based network size did not change between the types of interaction for a given treatment, but, in all cases, it was higher after 21 dpi (Table 3). The properties network degree, modularity and assortativity varied between treatments and types

TABLE 3 | Properties for each constructed network.

Properties	Template type	Network type	Groups of samples (treatments)	
			Pre-challenge and FMM bath	21 dpi
Network size	cDNA	Overall	109	111
		Co-occurrence	109	111
		Co-exclusion	109	111
	DNA	Overall	94	90
		Co-occurrence	94	90
		Co-exclusion	70	90
Network degree	cDNA	Overall	1514	2516
		Co-occurrence	1227	1547
		Co-exclusion	287	969
	DNA	Overall	923	1524
		Co-occurrence	698	962
		Co-exclusion	225	562
Modularity	cDNA	Overall	0.237	0.311
		Co-occurrence	0.392	0.311
		Co-exclusion	0.511	0.357
	DNA	Overall	0.463	0.294
		Co-occurrence	0.641	0.624
		Co-exclusion	0.459	0.458
Assortativity degree	cDNA	Overall	0.500	0.265
		Co-occurrence	0.614	0.752
		Co-exclusion	−0.490	−0.555
	DNA	Overall	0.575	0.005
		Co-occurrence	0.671	0.699
		Co-exclusion	−0.428	−0.593
Degree distribution	cDNA	Overall	Exponential	Exponential
		Co-occurrence	Exponential	Exponential
		Co-exclusion	Exponential	Exponential
	DNA	Overall	Exponential	Exponential
		Co-occurrence	Exponential	Exponential
		Co-exclusion	Exponential	Exponential

of interaction for DNA- and cDNA-based networks, respectively. An exception was the similarity in modularity between cDNA-based networks at 21 dpi (Table 3). In addition, overall and co-occurrence networks had positive values of assortativity in the pre-challenge state, while the co-exclusion network had negative values for this property. The same pattern was found at 21 dpi, but the overall network had an assortativity value close to zero (Table 3). No changes in degree distribution between and type of interactions (i.e., overall, co-occurrence and co-exclusion networks) were detected for either cDNA- or DNA-based networks (Table 3).

Taxa that were in the top 5% of the keystone index (i.e., the most important taxa) were determined (Tables S5 and S6). The fish pathogen *T. dicentrarchi* was in the top 48% and 49% of the most relevant taxa detected in samples from the pre-challenge state/FMM bath treatment in DNA- and cDNA-based networks (Figure 4A,C), respectively. Similarly, the bacterium was in

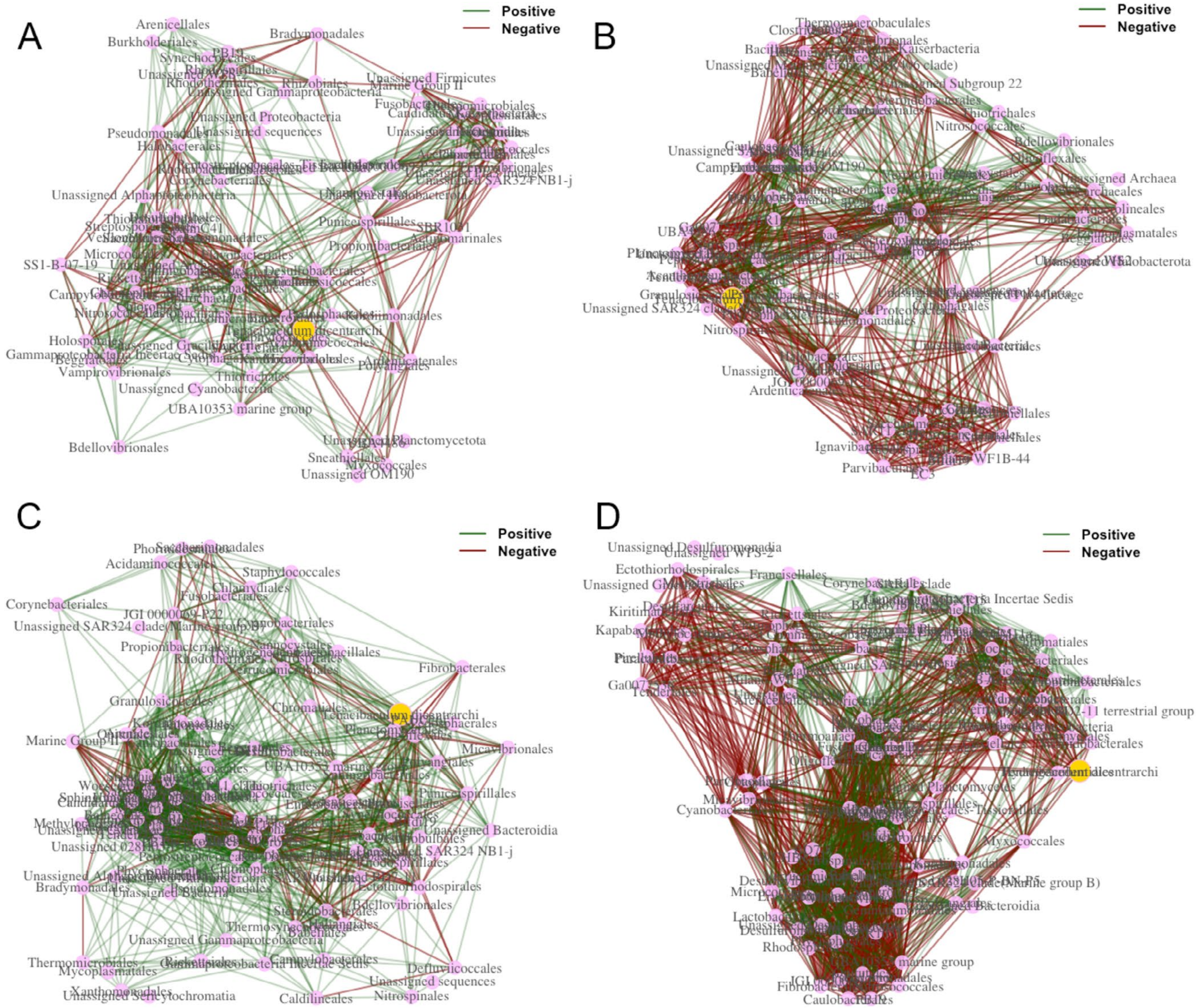

FIGURE 4 | Skin-associated microbiome network depicting interactions by treatment and type of template. Each circle represents a node and each link an interaction in networks constructed for (A, C) pre-challenge/FMM bath and (B, D) 21 dpi treatments, and from (A, B) DNA and (C, D) cDNA templates. Green and red links represent co-occurrence and co-exclusion interactions, respectively. *Tenacibaculum dicentrarchi* is represented by a yellow circle.

the top 40% of the most relevant taxa in samples from 21 dpi in DNA-based networks (Figure 4B), but in cDNA-based networks for samples at 21 dpi, *T. dicentrarchi* appeared only in the top 86% of the most relevant taxa (Figure 4D).

4 | Discussion

4.1 | Mortality of the Bath Challenge

The TdCh05 strain caused a cumulative mortality of 57.14% in Atlantic salmon specimens exposed to the bacterium, a value very close to the 65% cumulative mortality previously reported for the same bacterium and fish species (Avendaño-Herrera et al. 2016). In addition, we confirmed through PCR analysis of DNA from bacterial colonies as well as internal organ tissues that *T. dicentrarchi* was responsible for the clinical signs consistent with those described for tenacibaculosis (Avendaño-Herrera et al. 2016; Mabrok et al., 2023). It is important to note that neither the control fish nor those bathed in FMM broth (without bacteria) exhibited any mortality or macroscopic lesions. Based on these results, the study proceeded with skin samples from apparently healthy Atlantic salmon (i.e., no mortality or macroscopic lesions).

4.2 | Variability of Atlantic Salmon Skin-Associated Microbiome

Proteobacteria, Bacteroidota, Campylobacterota, Cyanobacteria, Patescibacteria and Verrucomicrobiota were among the top 10 phyla in different groups of skin-associated microbiome samples (Figure 1). All of these, plus Actinobacteriota, were also dominant in the pre-challenge state and after the FMM bath treatment.

Other authors have reported the dominance of Proteobacteria and Bacteroidota (formerly Bacteroidetes) in the skin microbiome of Atlantic salmon in seawater (Lokesh and Kiron 2016; Lorgen-Ritchie et al. 2022), as well as an increased relative abundance of members of Verrucomicrobia, Cyanobacteria (Lokesh and Kiron 2016) and Actinobacteria (Lorgen-Ritchie et al. 2022). In addition, members of Nanoarchaeota, Halobacterota and Thermoplasmatota (including unassigned archaea) were also detected in the skin-associated microbiome of Atlantic salmon (Figure 1). All averaged between 0.0%–0.026% of the total ASVs detected in microbiome samples collected from the pre-challenge state (and after the FMM bath). It has been reported that Halobacterota and Thermoplasmatota, as well as Thaumarchaeota, are the dominant archaeal components in coral-reef-fish skin microbiomes, with a cumulative abundance of approximately 0.8% determined by 16S DNA-based amplicon sequencing (Chiarello et al. 2018). This value is similar to the average cumulative abundance of Archaea (~0.12%) in our pre-challenge microbiome samples (according to 16S DNA-based amplicon sequencing). Few studies have reported the presence of archaea in fish skin microbiomes (Berggren et al. 2022), and there are no reports of archaeal detection in Atlantic salmon skin. Indeed, studies on fish mucosal microbiomes have been mostly focused on the gut and have left aside archaeal diversity, particularly in mucosal skin (Arun and Midhun 2023).

Previous studies comparing ulcerated and healthy fish reported significant changes in skin microbiome diversity in Atlantic salmon induced by different infections (Llewellyn et al. 2017; Valenzuela-Miranda et al. 2024; Coca et al. 2023; Godoy et al. 2023). The detected differences in compositional clustering per treatment (Figure 3A) were accompanied by variations in alpha diversity (Figure S1). For instance, some of the major species subjected to diversity loss at 2 hpi included the cold-adapted Gram-negative bacterium *Colwellia polaris* (Zhang et al. 2008, 2018), *Neptuniibacter marinus* (first isolated from *Pecten maximus* hatchery water) (Diéguez et al. 2017) and the Gram-negative hydrocarbon-degrading bacterium *Oceaniserpentilla haliotis* (Summers et al. 2024) (Figure 2A). ASVs belonging to the genera *Aurantivirga* (associated with copiotrophic bacteria) (Brüwer et al. 2023), *Spongiibacter*, *Amylibacter* (associated with potential biofilm-forming bacteria) (Oberbeckmann and Labrenz 2020) and *Polaribacter* (marine bacteria with various commensal species of marine fauna) (Bowman 2018) also experienced a loss of diversity or decreased abundance (Table S3). Interestingly, some members of *Spongiibacter* have shown inhibitory effects on a number of pathogenic fungi and bacteria (Hu et al. 2024), suggesting that the loss of *Spongiibacter* ASVs could lead to reduced host fitness in our study. Indeed, after *Tenacibaculum*, ASVs from *Spongiibacter*, *Polaribacter*, *Aurantivirga* and *Amylibacter* contributed importantly to the differences in compositional clustering patterns between samples from the pre-challenge state and those from TdCh05-challenged fish at 2 hpi (Table S3).

Increases in pathogen abundance and major changes in taxa composition have been proposed as signatures of dysbiosis in aquaculture fish microbiomes (Xavier et al. 2024). For example, infections with *Lepeophtheirus salmonis* (salmon louse) have been linked to an increase in alpha diversity and a profound destabilisation of the mucosal skin microbiome composition in Atlantic salmon (Llewellyn et al. 2017). In the present study, a strong increase in the abundance of *T. dicentrarchi* TdCh05 (Figure 2) and a significant decrease in alpha diversity were detected in the salmon skin-associated microbiome at 2 hpi (Table 2) (Figure S1). These changes were associated with an increase in the total number of *Tenacibaculum* ASVs, which contributed to between 53% and 58% of the differences in compositional clustering between samples from non-infected and TdCh05-challenged fish after 2 hpi (Tables S3 and S4). No significant differences in alpha diversity were detected between control and TdCh05-challenged fish at 21 dpi, or between these two groups of samples and those from the pre-challenged state or FMM bath (Figure S1). There were, however, important differences in taxa composition of the skin microbiome between TdCh05-challenged fish at 21 dpi and fish from the pre-challenged state or FMM bath (or even TdCh05-challenged fish at 2 hpi) (Figure 3A). Taken together, these results confirm that variations in alpha diversity as a criterion for dysbiosis should be taken with caution (Xavier et al. 2024), as *T. dicentrarchi* induced a significant decrease in alpha diversity only during the first hours after infection onset, a pattern that was not sustained over the course of the 21-day infection. Indeed, it is important to note that changes in skin microbial composition may be accompanied by a variable response in alpha diversity, depending on the species of fish infected and the etiological agent (Mougin and Joyce 2023). The drop in skin alpha diversity in TdCh05-challenged fish at 2 hpi was followed by an important detection of *Neptuniibacter marinus* at the end of the experimental time, both in infected and control fish (Figure 2). *Neptuniibacter* species are commonly detected in marine samples (Arahal et al. 2007), salt pans (Chen et al. 2012), or in association with marine organisms (Kudo et al. 2023), including the intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*) (Etyemez and Balcázar 2015). Particularly, *N. marinus* can also be isolated relatively easily from water and biofilm samples collected from recirculation systems (Diéguez et al. 2020). Considering the available information for *Neptuniibacter* spp. and our results, we cannot discard a potentially relevant role for *N. marinus* in skin microbiota homeostasis, representing a potential avenue for future research.

Widely used in the field of microbial ecology as a proxy for potential metabolic activity in environmental communities is 16S cDNA-based amplicon sequencing (e.g., Hoshino and Inagaki 2013; Levipan et al. 2016; Cardoso et al. 2017). This approach has also been implemented in microbiome studies of diverse organisms (Belheouane et al. 2017; Woltyńska et al. 2023), including the skin microbiome of salmonids (Pardo et al. 2023). No significant differences in the alpha diversity of the Atlantic salmon skin microbiome were detected between cDNA and DNA samples per treatment (Figure S1) (Table 2). Conversely, a NMDS ordination grouped the samples into two distinctive clusters depending on the kind of template (Figure 3B), with significant differences in ASV composition between templates for detected clusters (Table 1). The 23% of ASVs that contributed to 50% of the differences between the two identified clusters (Table S2) belonged to the genus *Tenacibaculum*; other genera containing ASVs with major contributions to these differences were *Oleispira*, *Neptuniibacter*, *Amylibacter* and *Spongiibacter* (Table S2). For most ASVs belonging to these genera—including *Tenacibaculum*—the mean number of reads obtained from 16S cDNA-based amplicon sequencing was higher than the average

count of reads from DNA-based sequencing (Table S2). Other authors have pointed out that 16S cDNA-based amplicon sequencing is not a reliable measure for overall metabolic profiling of environmental microbial communities (Wang et al. 2023). We agree with this notion in general, but our results suggest that 16S cDNA-based amplicon sequencing could at least provide the relative activity of major components of microbial communities—in our case, members of *Tenacibaculum*, *Oleispira*, *Neptuniibacter*, *Amylibacter* and *Spongiibacter*. Further research is needed to determine how members of these last four genera influence infections and outbreaks of tenacibaculosis.

4.3 | Microbiome Network Analysis

Although several infectious diseases in fish can lead to organ-related dysbiosis (e.g., skin, gills and gut) (Wynne et al. 2020; Rosado et al. 2023), the complexity of organ microbiomes in aquatic species could increase with disease progression (Dai et al. 2020). In our study, no important changes in network size were detected in general (Figure 4), but network degree (i.e., the mean number of interactions) indicated a higher complexity of DNA- and cDNA-based networks at 21 dpi when compared to networks from the pre-challenge state and FMM bath together (Table 3). Modularity has been positively associated with microbiome stability by affecting diverse community factors (e.g., habitat preferences and/or shared ecological functions) (Hernandez et al. 2021). This property showed an opposite trend between treatments in overall networks, depending on whether it was based on DNA or cDNA (Table 3). However, modularity in cDNA-based networks at 21 dpi was in general lower (compared to pre-challenge and FMM bath states) and similar between different types of networks. This suggests that the cDNA-based approach could be more sensitive for capturing the network stability at 21 dpi, as lower modularity can be associated with microbial network instability under disturbances (Wang et al. 2016; de Vries et al. 2018). In addition, assortativity has been positively correlated with network robustness (Kasthurirathna et al. 2013; Oña and Kost 2022). Results indicated that skin-associated microbiomes tended towards assortativity (based on node degree) in DNA- and cDNA-based networks, except for the DNA-based overall network at 21 dpi, which showed non-assortativity. In addition, disassortativity in all DNA- and cDNA-based co-exclusion networks, when high- and low-degree nodes tend to connect, was a reflection of potential suppression links between nodes.

Taxonomic groups belonging to *Vibrionaceae*, *Flavobacteriaceae*, *Pseudomonadaceae*, *Piscirickettsiaceae*, Rickettsial-like organisms and Peptostreptococcales-Tissierellales, which include pathogens or disease-related bacteria, are frequently detected on a range of aquatic hosts during coinfection processes (Llewellyn et al. 2017; Cruz-Flores and Cáceres-Martínez 2020; Rosales et al. 2023). There are various examples where *Tenacibaculum* spp. have been detected as part of fish coinfection stages. Particularly, *T. dicentrarchi* has been detected on the skin and/or gills in co-occurrence with diverse pathogens such as *L. salmonis* (Llewellyn et al. 2017), *Caligus rogercresseyi* (Morales-Rivera et al. 2022), *Neoparamoeba perurans* (Slinger et al. 2020) and *P. salmonis* (Godoy et al. 2023). In our study, *T. dicentrarchi* was detected at 21 dpi in co-occurrence with *Thiotrichales* in cDNA-based networks, with members such as *Thiothrix* spp., which have been associated with lesions in shrimp aquaculture (Karunasagar et al. 2004). In DNA-based networks at 21 dpi, *T. dicentrarchi* was detected in co-occurrence with members of Peptostreptococcales-Tissierellales, Rickettsiales, Pseudomonadales and Enterobacterales. Affiliated to this later order, representatives of *Vibrionaceae* were detected in co-occurrence with *T. dicentrarchi* in all networks. Finally, the detection of *T. dicentrarchi* after the FMM bath supports the idea that *Tenacibaculum* spp. could be a constant component in salmon smolt microbiomes (Slinger et al. 2020; Wynne et al. 2020; Godoy et al. 2023).

5 | Conclusions

T. dicentrarchi TdCh05 affected the skin-associated microbiome of Atlantic salmon in two ways over a 21-day infection. The early response in challenged fish at 2 hpi involved changes in skin-microbiome composition and a significant decrease in alpha diversity, while the second response at 21 dpi also showed important changes in microbiome composition, but a recovery in alpha diversity. These fluctuations indicated a short-term dysbiosis induced by *T. dicentrarchi* in the skin-associated microbiome of Atlantic salmon that was delimited within the first hours or days post-infection. Microbiome changes at 21 dpi were related to higher interaction complexity among taxa and community instability, as indicated by the network degree of all networks and the lowest modularity of cDNA-based networks, respectively. Ultimately, all these changes were associated with the death of challenged fish. Our results suggest that the infection process was related to the variability of members of the genera *Oleispira* (Pseudomonadales), *Neptuniibacter* (Pseudomonadales), *Amylibacter* (Rhodobacterales), *Polaribacter* (Flavobacteriales) and *Spongiibacter* (Pseudomonadales), the so far unknown (direct or indirect) roles of which in tenacibaculosis may be key to the onset and/or progression of the disease. Taken together, microbiome analyses suggest that members of Pseudomonadales could be important accompanying microorganisms of *T. dicentrarchi* during disease progression.

Author Contributions

Ruben Avendaño-Herrera: conceptualization, visualization, writing – original draft, supervision, project administration, writing – review and editing, funding acquisition, resources, validation. **Linette Tralma:** data curation, formal analysis, investigation, methodology, software, writing – original draft. **Hernán Wicki:** investigation, methodology. **Fernanda Barrios-Henríquez:** investigation, methodology. **Héctor A. Levipan:** conceptualization, visualization, writing – original draft, supervision, project administration, writing – review and editing, funding acquisition, resources, validation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data supporting the findings are available upon request from the corresponding author.

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Supporting Information

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