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Posted Date: 27 November 2024

doi: 10.20944/preprints202411.2156.v1

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Tenacibaculum Virulence and Resistance: Genomic Insights

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Abstract: Tenacibaculosis, an ulcerative skin disease induced by members of the genus Tenacibaculum (Family Flavobacteriaceae, phylum Bacteroidetes), presents significant economic challenges for the global salmon industry. This research specifically examines *Tenacibaculum dicentrarchi*, a prominent pathogen linked to tenacibaculosis outbreaks in Coho salmon (*Oncorhynchus kisutch*). The prevalence of tenacibaculosis, attributed to *T. dicentrarchi, T. maritimum, and Tenacibaculum finnmarkense,* has been noteworthy in Chile, with considerable annual mortality rates documented in recent years. To shed light on the virulence and antibiotic resistance mechanisms of *T. dicentrarchi,* we conducted a genomic analysis of two isolates obtained from Atlantic salmon. Prior studies have explored virulence-related genes, pathogenicity factors, and mechanisms of iron acquisition and copper homeostasis in four isolates of *T. dicentrarchi* from Atlantic salmon. Additionally, resistance to tetracycline and fluoroquinolones has been investigated. However, a comprehensive understanding of variations in virulence genes and antibiotic resistance mechanisms among different isolates remains limited. In our investigation, we present a detailed analysis of the genomes of the two *T. dicentrarchi* isolates, focusing on identifying key virulence factors and antibiotic resistance determinants. This research will contribute to a deeper understanding of the pathogenicity and antimicrobial resistance mechanisms exhibited by *T. dicentrarchi*, shedding light on potential therapeutic targets for the management of tenacibaculosis in salmonids.

Keywords: whole-genome sequencing; *T. dicentrarchi*; virulence factors; resistance factors

1. Introduction

Tenacibaculum spp. is the etiological agent of Tenacibaculosis an ulcerative skin disease that affects so many fish species in the world, this pathogen produces clinical symptoms that acutely progress and trigger death (Avendaño-Herrera et al. 2020), (Krkosek et al. 2024). Its clinical signs are observable after transfer to seawater, highlighting the presence of macroscopic lesions such as ulcers and necrosis on the surface of the body, eroded mouth, frayed fins, and rotted tail, we can observe necrosis on the gill, in eyes we can find choroidal congestion and sub-choroidal hemorrhage, sometimes with eye rupture (Ostland, Morrison, y Ferguson 1999a) and (Irgang y Avendaño-Herrera 2021), (Avendaño-Herrera, Lopez, et al. 2024). The genera belonging to the Flavobacteriaceae family, these bacteria are as strict aerobic bacilli with negative Gram stain, with motility, and can be grown

 doi:10.20944/preprints202411.2156.v1

in marine agars. This pathogen family is able to grow in four different kinds of agar medium, including Marine Shieh's Selective Medium (Kumanan et al. 2022) and Flexibacter maritimus medium (Pazos et al. 1996). Their growth temperature varies depending on the species, with optimal temperatures for *T. discolor* and *T. gallaicum* between 14-38°C and for *T. finnmarkense* from 2°C to 20°C (Fernández-Álvarez y Santos 2018).

In Tenacibaculum genus, at this moment describes species associated with fish include T. bernardetii, recently proposed by Avendaño-Herrera, Saldarriaga-Córdoba, y Irgang 2023, T. dicentrarchi, T. discolor, T. finnmarkense (Småge, Brevik, et al. 2016) , T. maritimum, T. soleae, T. ovolyticum and T. piscium (Michnik et al. 2024), (Avendaño-Herrera et al. 2022), which has been isolated from wild, cultured anadromous, and marine fish species, such as salmonid species (A. B. Olsen et al. 2017), (Valdes et al. 2021), Atlantic cod (Gadus morhua) (Avendaño-Herrera et al. 2004), red conger eel (Genypterus chilensis) (Saldarriaga-Córdoba, Irgang, y Avendaño-Herrera 2021), Atlantic herring (Clupea harengus), Atlantic halibut (Hippoglossus hippoglossus), lumpfish (Cyclopterus lumpus) and shark (Carcharias taurus) (Florio et al. 2016). This bacterium (Tenacibaculum spp) has a wide geographical distribution: Europe (Småge, Frisch, et al. 2016) and (Bernardet, Kerouault, y Michel 1994), Asia (Baxa 1988), Oceania (Wilson, Douglas, y Dunn 2019a), and North and South America (Ostland, Morrison, y Ferguson 1999b). Specifically, in Atlantic salmon (Salmo salar), tenacibaculosis has been reported under various names and associated with different species in several countries, including the United States (Frelier et al. 1994), Australia (Handlinger, Soltani, y Percival 1997) , Canada (Ostland, Morrison, y Ferguson 1999c), Norway (A. Olsen et al. 2011), and Chile (Avendaño-Herrera et al. 2016a). In Chile according to the records of the National Fisheries and Aquaculture Service (Sernapesca), the morbi-mortality of tenacibaculosis in salmonids in Chile is mainly caused by T. dicentrarchi and T. maritimum (Wakabayashi, Hikida, y Masumura 1986), it has been secondary annual mortalities during 2019, 2020, and 2021 corresponded to 14, 25, and 31% for the Atlantic salmon (Salmo salar) species according with the National Fisheries and Aquaculture Service of Chile(Sernapesca).

Genomic characterization has been established in various countries. Analysis of the 16S ribosomal RNA shows different genogroups, which may present varying degrees of virulence, leading to high mortality or affecting specific species.

The establishment of an infectious disease is triggered by: i) environmental changes, which increase the likelihood of the establishment of new pathogens (Wade et al. 2019) ii) high levels of overcrowding in farming systems, which produce high levels of stress, making fish more susceptible to pathogen outbreaks and iii) aquaculture massification (Manley et al. 2014) and (Ellis et al. 2002), but what factors of *Tenacibaculum* allow its success? Some studies evidence endemic colonization of aquaculture systems and parallel evolution of fish pathogenicity (Habib et al. 2014), (Bellec et al. 2024), (Coca et al. 2023); however, there are many uncertainties about the specific virulence factors present in this bacterium. To address this, we have examined four strains of *T. dicentrarchi* isolated from Atlantic salmon (*S. salar*) to identify virulence genes. Specifically, we are investigating mechanisms of iron acquisition, regulation of copper levels, resistance to tetracycline and fluoroquinolones, as well as pathogenicity IG (Avendaño-Herrera, Echeverría-Bugueño, et al. 2024), (Saldarriaga-Córdoba, Irgang, y Avendaño-Herrera 2021). Despite the valuable insights gained, significant gaps remain regarding the differences in virulence genes and antibiotic resistance mechanisms among various isolates. To address this, we analyzed the genomes of two *Tenacibaculum dicentrarchi* isolates from Atlantic salmon (*Salmo salar*), focusing on identifying the primary virulence and antibiotic resistance factors. This analysis provides a deeper understanding of the mechanisms underlying virulence and antimicrobial resistance in *T. dicentrarchi*.

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2. Materials and Methods

2.1. Sample Collection and Gross Pathology

The collected fish presented morbidity and mortalities, evidencing severe body injuries like the clinical signs caused by *T. dicentrarchi*. In the case of Atlantic salmon, all were confirmed as positive for *T. dicentrarchi* by isolation and qPCR. A total of 2 tenacibaculosis samples were obtained for the current study. These isolates were collected as part of two ongoing Chilean surveillance programs. The first sample was derived from the General Sanitary Mortality Management Program (PSGM), which involves companies and private laboratories conducting passive surveillance of diseases based on sanitary and productive criteria. Fish displaying at least one of the specified symptoms were chosen for sampling. The provided records thoroughly detailed the observed clinical signs, including fin erosion and ulceration, lesions in the buccal, opercular, and rostral areas, as well as occurrences of branchial necrosis and yellow pigmentation.

2.2. Bacterial Strains and Growth Conditions

To isolate bacteria, we collected samples from both the external tissues and internal organs (such as the kidney, and liver) of each fish. These samples were then streaked onto plates containing Flexibacter maritimus medium (Pazos et al., 1996). The plates were subsequently incubated at 18 ºC for 7 days.

2.3. Histopathology

Tissue specimens intended for histological examination were preserved in 10% buffered formalin. Subsequently, standard procedures were employed for their processing, and sections measuring 3–4 µm were stained with hematoxylin and eosin (H&E), following the methodology outlined by Prophet et al. (1992), to elucidate microscopic morphological alterations.

2.4. DNA Extraction and Sequencing and Library Construction

The culture of Tenacibaculum strains was isolated from Atlantic salmon (*Salmo salar*) in a marine agar medium at a temperature of 18 °C. Gram staining was performed, obtaining Gram-negative filamentous bacteria. DNA extraction was performed using the TANBead Nucleic Acid Extraction kit (M61GS46) by mechanical extraction using the Automatic Nucleic Acid Extraction robot MaelstromTMMaelstromTM4800. Two colonies of the cultured strains were placed on the marine agar in sterile PBS. Colonies were vortexed for 30 seconds. They are centrifuged for 5 minutes at 13,000 rpm. Once the pellet is obtained, 200 µl of incubation buffer and 10 µl of proteinase K are added and incubated at 56 °C for 60 minutes. The lysate is transferred to the 1/7 column. We are obtaining 120 µl of extraction. We measured DNA concentration using 2 µl of samples through the Invitrogen Qubit dsDNA BR Assay kit (Invitrogen Qubit 4 Fluorometer). Following the Nextera XT DNA Library Prep® protocol, isolated DNA sequencing was used for whole genome sequencing.

The library amplification was performed using the Nextera XT DNA Library Prep kit under the following conditions: 72 °C for 3 minutes, 95°C for 30 seconds, followed by 12 cycles of 95 °C for 10 seconds; 55 °C for 30 seconds and 72 °C for 5 minutes. After that, a final elongation step was performed at 72 °C for 5 minutes. Next, all samples were indexed using IDT for Illumina Nextera UD Indexes Set B and cleaned up. Samples were sequenced using the Illumina Miniseq platform, following the manufacturer's protocols (Illumina DNA prep kit and Nextera Library XT DNA preparation kit).

2.5. Quality Control and Genome Assembly

Quality control of raw reads was assessed with FastQC (De Sena Brandine y Smith 2021) and summarized with MultiQC (Ewels et al. 2016). Trimming of reads was performed with Trimmomatic (Bolger, Lohse, y Usadel 2014), de novo genome assembly was accomplished with SPAdes (Bankevich et al. 2012) and assessed with QUAST (Gurevich et al. 2013).

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2.6. Phylogenetic Analysis

Tenacibaculum dicentrarchi and *Tenacibaculum finnmarkense* genomes were retrieved from NCBI. SNPs were extracted from genomes sequenced in this study and from genomes obtained from NCBI, and a SNP multiple sequence alignment was constructed with PhaME (Shakya et al. 2020). A phylogenetic tree was built under maximum likelihood from 146,782 SNP positions in the core genome with RAxML (Stamatakis 2014). Whole-genome Average Nucleotide Identity (ANI) was computed between all pairs of genomes with FastANI (Hernández-Salmerón y Moreno-Hagelsieb 2022).

2.7. Virulence and Resistance Factors Analysis

Genomes assembled in this study and genomes retrieved from NCBI were annotated with Prokka (Seemann 2014). Virulence factors (VF) were identified by performing local alignments with Protein BLAST (Altschul et al. 1990) , were query sequences corresponded to translated CDS sequences obtained from Prokka, and the VFDB protein sequences of full dataset (Liu et al. 2019) were used as database. Similarly, antibiotic resistance (AR) genes were found as above but using the CARD (McArthur et al. 2013) database. In both cases, blastp hits with at least 0.0001 e-value, 50% of similarity, and 60% query coverage were considered for downstream analysis.

3. Results

3.1. Clinical Sign and Bacterial Isolation

Examination of smears obtained from skin lesions disclosed a plentiful presence of rod-shaped, elongated Gram-negative bacteria. At the tissue level, filamentous structures with a multifocal distribution were identified on the muscle surface, and these were determined to be consistent with bacteria belonging to the genus Tenacibaculum sp. (Figure 1).

All fish mainly presented external macroscopic lesions (e.g. skin lesions, tail rot, and hemorrhagic mouth) in different body parts, but mainly erosion and loss of upper and lower mandibular tissue, with high yellow pigmentation, were observed. (Figure 2). being the typical lesions of tenacibaculosis.

3.2. Pan-Genome Characterization

The PIRATE toolbox was applied to 318 *Tenacibaculum* genomes, from which 4 strains are reported in this study and 314 were retrieved from NCBI [Sayers et al., 2022]. The pangenome of the *Tenacibaculum* genus comprised 41079 gene families, of which 1243 (3.03%) were classified as core (>95% genomes) and 39836 (96.97%) as accessory. The analysis of the present/absent gene families showed remarkably distinct patterns through the *Tenacibaculum* genus, especially between salmonid and non-salmonid affecting *Tenacibaculum* species (Figure 3).

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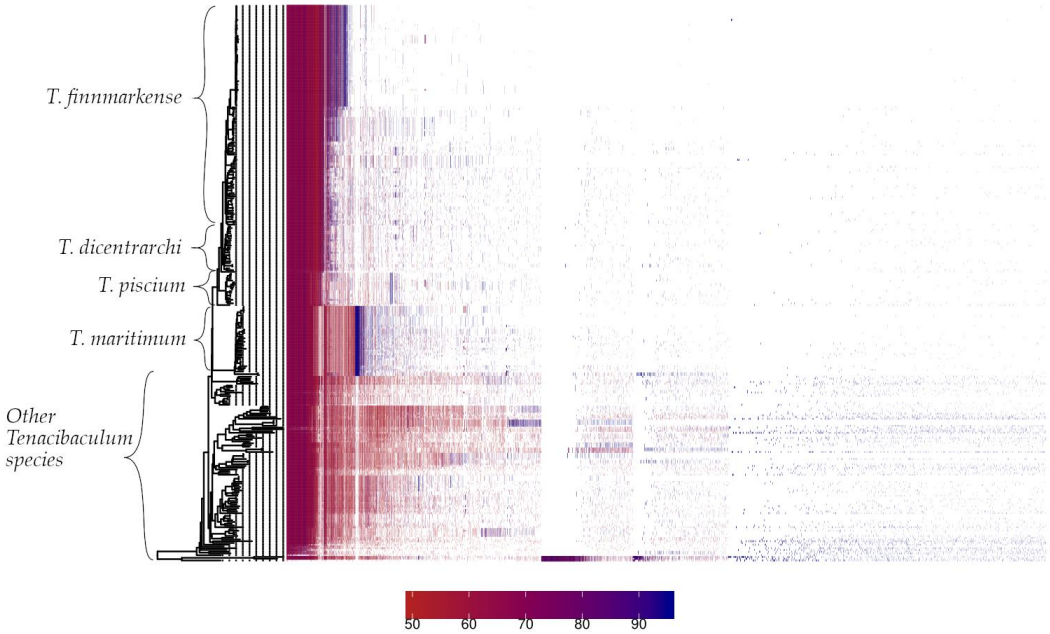

Figure 3. Pan-genome analysis of the *Tenacibaculum* genus (n = 318). Shared gene presence per isolate, ordered alongside the phylogenetic tree. Gene family presence is indicated by colored blocks per column, reflecting the corresponding percentage identity thresholds.

3.3. Phylogenomics

The analysis focused on 138 genomes of salmonid-affected species. A maximum likelihood dendrogram was generated to show the grouping of these species (refer to Figure 4). In the case of *T. finnmarkense*, strains affecting salmonids grouped into two clusters, one of which further divided into two subclusters (highlighted in green). Out of 82 *T. finnmarkense* isolates, 80 were from *S. salar*, and 2 were from *O. mykiss*. Meanwhile, strains of *T. piscium* affecting salmonids grouped into a single cluster. Out of the 15 *T. piscium* isolates, 11 were from *S. salar*, 2 from *O. kisutch*, and two from *O. mykiss*. Notably, all other *Tenacibaculum* genomes affecting salmonids, including *T. dicentrarchi*, were isolated from *S. salar* exclusively.

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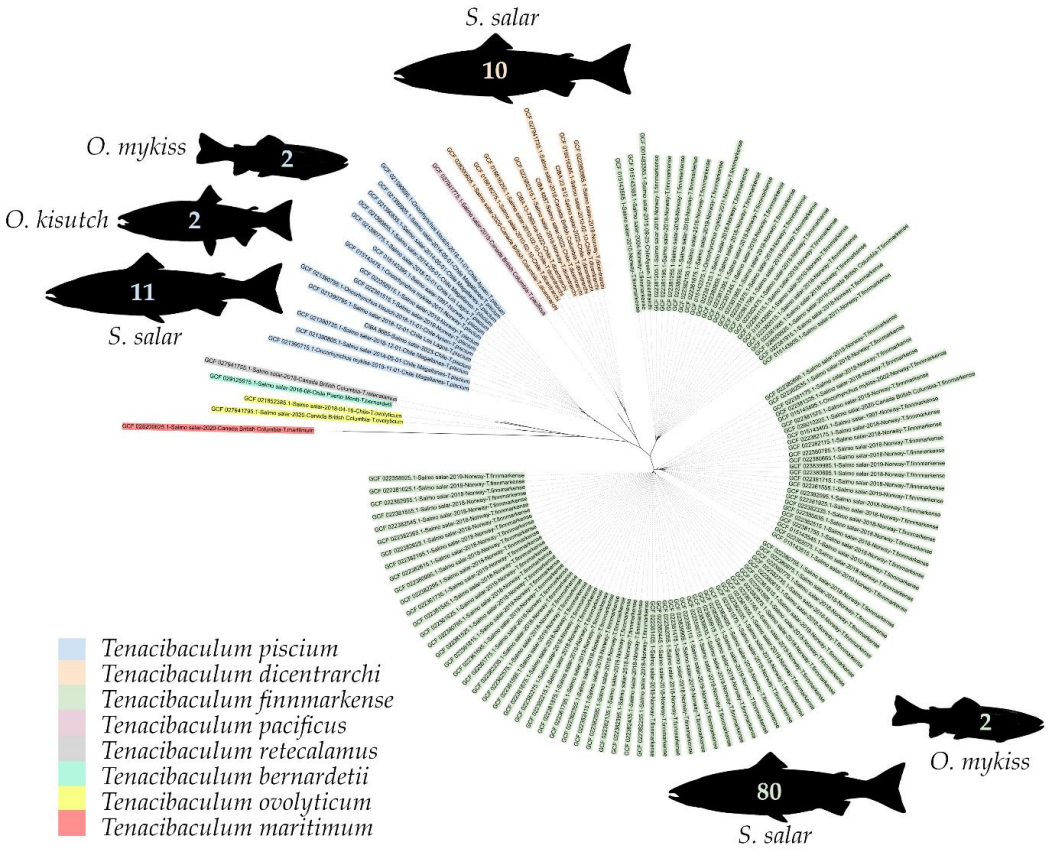

Figure 4. Phylogenomic analysis of the *Tenacibaculum* species affecting salmonids (n = 138). Maximum likelihood phylogenetic tree constructed from a core genome alignment. *Tenacibaculum* species are indicated in colors. Sample sizes of corresponding salmonid host species are highlighted.

3.4. Virulence Factors

Virulence factors (VFs) were identified through pangenome analysis of gene families. A total of 641 VFs were found in *Tenacibaculum* genomes associated with hosts. Out of these, 45 were exclusively present in isolates affecting salmonid fish (see Figure 5A). Interestingly, most of these VFs specifically affecting salmonid fish are linked to immune modulation (see Figure 5B). Moreover, each *Tenacibaculum* species affecting salmonid fish exhibits a unique set of immune modulation VFs, with *T. retecalamus* containing the highest number of such VFs (see Figure 5C). The list of these 45 VFs only found in salmonid-affecting isolates, along with their similarity percentages, is available in Supplementary Table S1.

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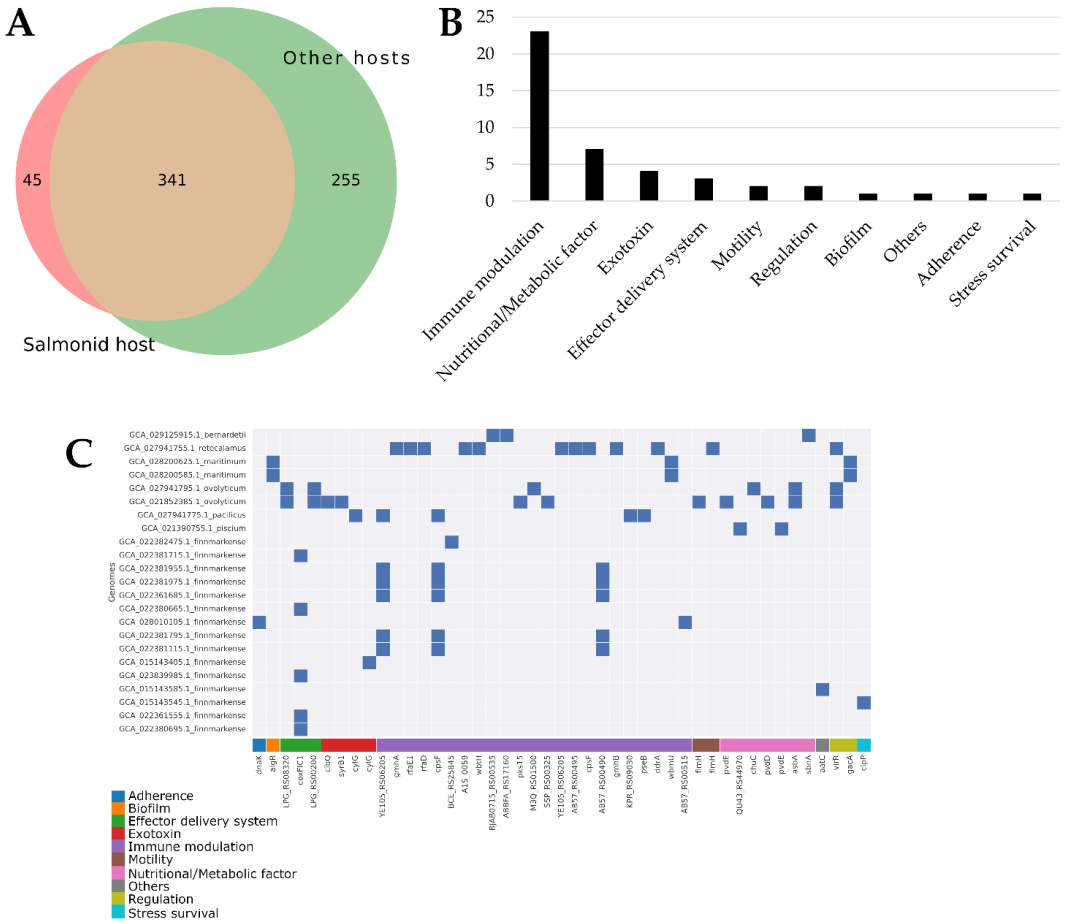

Figure 5. Only salmonids affecting virulence factors (VFs) profile among *Tenacibaculum* species. **A**: Presence of VFs among salmonids and other hosts. **B**: Frequency of only salmonids affecting VFs by category. **C**: Presence/absence matrix of only salmonids affecting VFs among *Tenacibaculum species* by category.

3.5. Antibiotic Resistance Factors

The pangenome analysis identified gene families containing Antibiotic Resistance factors (ARs). 126 ARs were found in the *Tenacibaculum* genomes associated with hosts. Among these, 81 ARs were present in isolates affecting salmonids, and 6 of these were unique to salmonid-affecting isolates (see Figure 6A). The 81 ARs found in salmonid-affecting isolates included factors related to tetracycline, phenicol, and other drug families (see Figure 6B). Antibiotic efflux is the most identified mechanism among these factors (see Figure 6C). More details about the 6 and 75 ARs present in salmonid-affecting isolates and their similarity percentages can be found in Supplementary Tables S2a and S2b.

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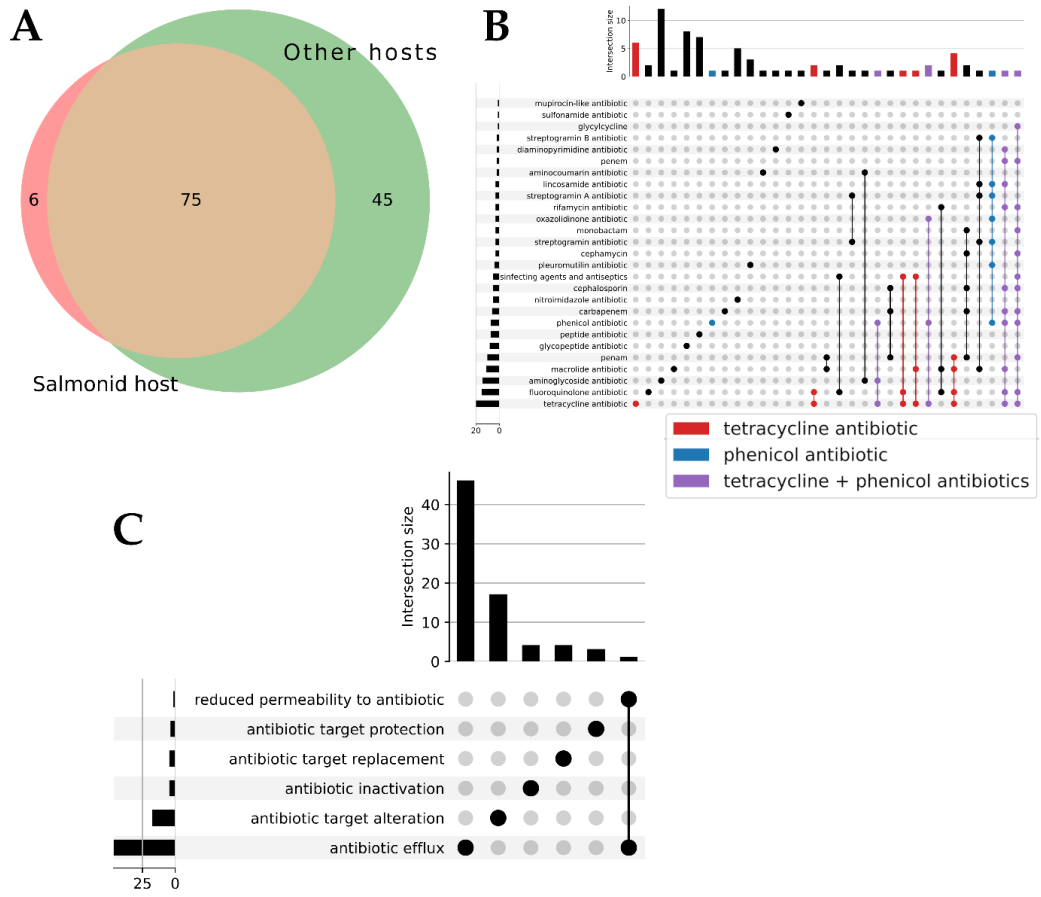

Figure 6. Antibiotic resistance factors (ARs) landscape among *Tenacibaculum* species. **A**: Presence of ARs among salmonids and other hosts. **B**: Frequency of ARs by affected drug family. **C**: Frequency of identified ARs mechanisms.

3.6. Restriction-Modification System Abundance

Predicted Restriction-Modification System coding sequences (RMs) were identified through pangenome analysis of gene families. A total of 93 RMs were found in *Tenacibaculum* genomes associated with hosts. Among these, 14 were exclusive to isolates affecting salmonids (see Figure 7A). Notably, type I methyltransferase is the most prevalent RM found only in salmonid-associated isolates (see Figure 7B). The list of these 14 RMs exclusive to salmonid-affecting isolates is available in Supplementary Table S3.

 doi:10.20944/preprints202411.2156.v1

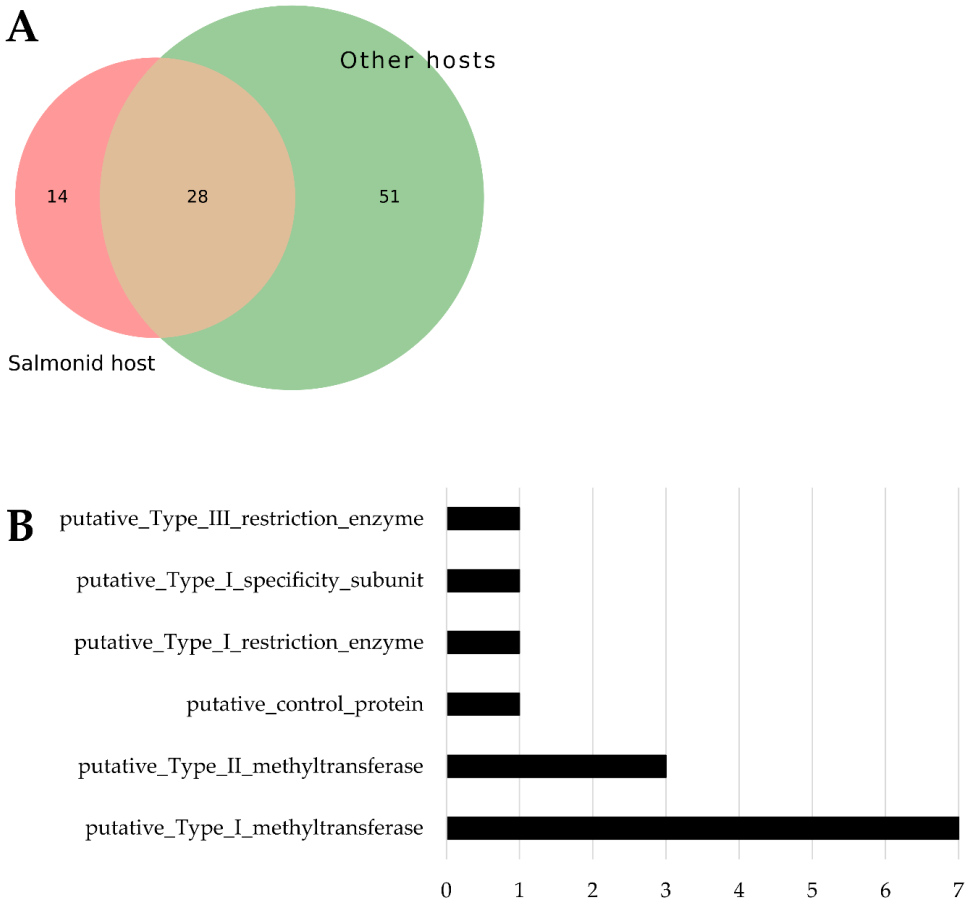

Figure 7. Restriction-Modification System coding sequences (RMs) profile within *Tenacibaculum* species. **A**: Presence of RMs among salmonids and other hosts. **B**: Frequency of only salmonids affecting RMs by category.

4. Discussion

Within the *Tenacibaculum* genus, several important fish-pathogenic species exist, including *T. maritimum, T. dicetrarchi,* and *T. finnmarkense*. In Chile, *T. dicetrarchi* shows a sustained increase in overall mortality due to cases of infectious tenacibaculosis, particularly in Atlantic salmon (*Salmo salar*) (SERNAPESCA, 2018; SERNAPESCA, 2021 (January-November) (Nowlan, Lumsden, y Russell 2021).

This bacterium was initially identified in Spanish sea bass (*Dicentrarchus labrax*) with damaged skin (Piñeiro-Vidal et al. 2012). Subsequently, *Tenacibaculum dicetrarchi* has been identified as the causative agent of disease outbreaks in Atlantic salmon (*Salmo salar*) and red conger (*Genypterus chilensis*) in Chile (Avendaño-Herrera et al. 2016b) and (Irgang y Avendaño-Herrera 2022a). Additionally, cases have been reported in Atlantic salmon (*S. salar*) in Norway (Klakegg et al. 2019) and (Olsen et al. 2017) and Tasmania (Wilson, Douglas, y Dunn 2019b). Norway and Scotland have also documented occurrences of this bacterium in Atlantic cod (*Gadus morhua*) and largemouth bass (*Cyclopterus lumpus*) (Olsen et al., 2017), as well as ballan fish (Papadopoulou et al. 2021). Despite the significant impact of *T. dicetrarchi* induced tenacibaculosis on Chilean aquaculture, there is limited knowledge about the bacterium's pathogenesis, infection pathways, and antibiotic resistance mechanisms.

Adherence of bacterial cells to fish tissue surfaces is crucial during the initial stages of Tenacibaculosis infection. Microscopic examinations of smears from ulcerative skin lesions commonly reveal abundant long rods and Gram-negative bacteria consistent with descriptions of Tenacibaculum cells (Figure 1). This bacterium induces severe external macroscopic skin lesions and necrosis, affecting various body surface areas, occasionally accompanied by bone exposure (Mabrok

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et al. 2023), (Echeverría-Bugueño et al. 2023), primarily in the maxillofacial and cranial regions, as depicted in Figure 2. Other external manifestations of the disease include reddening and erosion of the skin beneath the jaw, hemorrhagic mouth and operculum, and yellowish discoloration in the oral and dental areas (Ostland, Morrison, y Ferguson 1999) and (Irgang y Avendaño-Herrera 2021), (Avendaño-Herrera et al. 2023).

The phylogenetic and pangenomic analysis, derived from SNP sequences, reveals three distinct clusters in the phylogenetic tree. Notably, each of our three analyzed genomes is positioned within a separate cluster (Figure 3).

In our analysis, we have identified both core genes and accessory genes, allowing us to determine the number of homologous gene families in the sequences we studied. When we conducted a phylogenetic analysis of *T. dicetrarchi* sequences, we observed three distinct clusters (see Figure 3). Each of the isolates we sequenced belongs to a unique cluster. Upon examining virulence factor-expressing genes, we primarily found the presence of type III, IV, and V secretion systems. Among the isolates we scrutinized, only one exhibited a type II secretion system.

Notably, the Type III Secretion System (T3SS) emerges as significant, which potentially could be used as a mechanism for bacteria to release effector proteins into host cells, thereby enhancing virulence and colonization. This system has been reported as a pathogenicity mechanism (Notti y Stebbins 2016) for certain bacteria groups such as *Yersinia* and *Shigella*.

Among the identified genes is the pore-forming toxin (PFT) system, prevalent in the majority of analyzed sequences, except for one (Td_CIBA_08_2022). The absence of PFT in this sequence suggests a potential variance in cell invasion mechanisms. Genes responsible for encoding virulence factors were identified and categorized into subgroups, particularly associated with type II, III, IV, and V secretion systems, ionophore pore-forming toxins, genotoxins, cell invasion genes, antiphagocytic genes, and biofilm formation, as well as stress survival.

The goal of this comparative analysis is to offer insights into the genes and pathways that may be involved in the virulence mechanisms of *T. dicetrarchi*. The identified genes, either in whole or in part, could play a role in the organism's ability to cause disease in fish. To gain a more thorough understanding, further research is necessary into the physiological aspects and infectivity of these predicted genes.

Between late July 2018 and mid-August 2020, there were outbreaks of tenacibaculosis in marine aquaculture cycles, with a 52.5% prevalence rate (520 out of 990 cycles) (SERNAPESCA, 2020). Currently, there is no vaccine for this pathogen, requiring the use of antimicrobial compounds prescribed by veterinarians to manage and control the disease. In 2019, 6.2 tons of florfenicol (FFC) and 3.1 tons of oxytetracycline (OTC) were administered for tenacibaculosis control, accounting for 2% and 1%, respectively, of the total 311.2 tons of antimicrobials used in marine farms that year (SERNAPESCA, 2019b, 2020).

Our analysis identified several antibiotic resistance genes (ARGs), raising concerns, particularly about those associated with Florfenicol and Tetracyclines, the most widely used antibiotics in Chilean aquaculture (REF). Among the sequences analyzed, the tetracycline resistance genes tetA and tetB were found, with two isolates also exhibiting the tet(Q) gene. Additionally, resistance genes corresponding to commonly used antibiotics—such as sul4 for sulfonamides and cat86 for phenicols—were detected, including enrofloxacin, florfenicol, trimethoprim, and sulfadiazine (Nowlan et al., 2020; Rigos et al., 2020; Saldarriaga-Córdoba et al., 2021). In the absence of a commercial vaccine, antibiotic treatment remains the primary method to combat *Tenacibaculum* sp. in aquaculture, particularly since juvenile fish are occasionally exposed to tenacibaculosis in hatchery environments (Irgang y Avendaño-Herrera 2022b).

Bacteria use restriction and modification (R-M) systems as a defense mechanism to restrict the entry, integration, and replication of foreign genetic elements. These systems act like the innate immune system of bacteria, defending against foreign DNA (Oliveira, Touchon, y Rocha 2014). In addition to their role in defense, R-M systems can also limit sequences generated by genomic damage and process free DNA ends, influencing the fate of DNA acquired by the cell and maintaining the genetic boundaries of bacterial species (Rocha 2001). Although R-M system proteins are widely used

 doi:10.20944/preprints202411.2156.v1

in molecular biology, biotechnology, and biomedicine to modify DNA, their ecological aspects, including occurrence, distribution, diversification, and impact on microbial evolution through horizontal gene transfer of mobile genetic elements, are not fully understood.

5. Conclusions

The analysis of *Tenacibaculum* strains reveals significant genetic specialization in those affecting salmonid fish. These strains exhibit distinct gene profiles, including unique virulence factors primarily linked to immune modulation, with *T. retecalamus* showing the highest number. Additionally, they possess specialized antibiotic resistance genes, particularly against tetracycline and phenicol, suggesting an adaptation to survive treatments common in salmonid aquaculture.

Furthermore, these strains have abundant restriction-modification systems, especially type I methyltransferases, which may help them evade the host immune response or adapt to specific environmental conditions associated with salmonids. Overall, the genetic distinctions in these strains underscore their specialized evolution for infecting salmonid hosts.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, J.P.P.,M.G. ; methodology and study design, M.G., J.P.P., D.C, M.M and Y.C.; sampling, D.C. , bioinformatic analysis, M.M.; interpretation, Y.C., B.S., M.G. J.P.P and M.M.; writing—original draft preparation, M.G. J.P.P and M.M and Y.C.; writing—review and editing, M.G., J.P.P. and Y.C.; supervision, J.P.P. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Centro de Investigaciones Biológicas Aplicadas, grant number: clinical_genomic_Bacteria_200 and Fondecyt de Iniciación Nº 11230401 (J.P.P).

Data Availability Statement: Raw data from this study is available at NCBI. The Centro de Investigaciones Biológicas Aplicadas (CIBA) in Puerto Montt, Chile, holds certification for aquaculture diagnostic services. The Chilean National Fish and Aquaculture Service (Servicio Nacional de Pesca y Acuicultura de Chile, http://www.sernapesca.cl/, accessed on 10 November 2022) approved all associated experimental protocols. Our procedures were conducted in accordance with Chile's Ley 20.380 on animal protection, which governs animal welfare in biomedical research, and they closely followed the guidelines set out in the EU Directive 2010/63/EU on the protection of animals in scientific research.

Acknowledgments: We extend our gratitude to our colleagues in the Chilean salmon industry for permitting the collection of samples for this study. We deeply appreciate the technician and veterinary teams at the Centro de Investigaciones Biológicas Aplicadas (CIBA) for their invaluable support. Furthermore, we give our thanks to the Agencia Nacional de Investigación y Desarrollo de Chile (ANID) for funding J.P.P, Fondecyt de Iniciación Nº 11230401.

Conflicts of Interest: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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 doi:10.20944/preprints202411.2156.v1

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