



**HAL**  
open science

## **Occurrence of virulence factors in the *Vibrio* community associated with Pacific Oysters from farming sites in northern Chile**

Daniel Oyanedel, Felipe Navarro, Katherina Brokordt, Paulina Schmitt

### ► **To cite this version:**

Daniel Oyanedel, Felipe Navarro, Katherina Brokordt, Paulina Schmitt. Occurrence of virulence factors in the *Vibrio* community associated with Pacific Oysters from farming sites in northern Chile. *Aquaculture*, 2026, 613 (1), pp.743391. <10.1016/j.aquaculture.2025.743391>. <hal-05404771>

**HAL Id: hal-05404771**

**<https://hal.science/hal-05404771v1>**

Submitted on 8 Dec 2025

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

## Occurrence of virulence factors in the *Vibrio* community associated with Pacific Oysters from farming sites in northern Chile

Daniel Oyanedel <sup>a,\*</sup>, Felipe Navarro <sup>a</sup>, Katherina Brokordt <sup>b</sup>, Paulina Schmitt <sup>a</sup>

<sup>a</sup> Grupo de Biomarcadores de Holobiontes Marinos Acuícolas (BIHOMA), Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

<sup>b</sup> Laboratorio de Fisiología y Genética Marina (FIGEMA), Departamento de Acuicultura, Facultad de Ciencias del Mar, Universidad Católica del Norte, Larrondo 1281, Coquimbo, Chile

Coquimbo, Chile

\*Corresponding author E-mail address: [daniel.oyanedel@pucv.cl](mailto:daniel.oyanedel@pucv.cl) (D. Oyanedel).

### ABSTRACT

Infectious diseases are a major threat to sustainable aquaculture, yet the biosafety risks associated with emerging pathogens in farming sites are often overlooked, particularly when no mass mortality events are observed. In this study, we investigated the occurrence and virulence potential of *Vibrio spp.* within the microbiota of Pacific Oysters (*Magallana gigas*) farmed in Tongoy Bay, Chile. Using *in vitro* cytotoxicity assays, comparative genomics, and fluorescence microscopy, we characterized the cytotoxicity of *Vibrio* strains on oyster hemocytes and identified key virulence determinants. We found a positive correlation between virulence and cytotoxicity, with strains such as *V. lentus* TO6\_3 and *V. lentus* TO5\_9 causing significant hemocyte lysis. Whole-genome sequencing revealed the presence of diverse macromolecular systems, including Type VI secretion systems (T6SS) and a multifunctional auto-processing repeats-in-toxin (MARTX) cluster exclusive to the highly virulent but moderately cytotoxic *V. lentus* TO6\_11. This strain induced hemocyte rounding, a phenotype consistent with the presence of the actin crosslinking domain (ACD) and the makes caterpillar floppy (MCF) effector domain, probably linked to a mechanism of pathogenicity that does not completely rely on hemocyte lysis for the establishment of infection. The seasonal monitoring of virulence factors revealed a winter increase in T6SS frequency, while MARTX prevalence remained low. Our findings expose the silent circulation of virulence factors in oyster-associated *Vibrio* communities and highlight the need for targeted monitoring to prevent future disease outbreaks in emerging aquaculture systems ;

### KEYWORDS

*Magallana gigas* - Pathogen monitoring - Pathogenicity determinants - Aquaculture biosafety MARTX – toxin - Type VI secretion system

## 1. Introduction

Farming of bivalve species is a major food-producing industry, contributing approximately 14 % of global marine production, with an annual yield of 15 million tonnes (Wijsman et al., 2019). Marine bivalve species such as clams, oysters, mussels and scallops provide a sustainable source of high-quality food (Song et al., 2024) and promotes the development of local economies (van der Olivier et al., 2020). Chile leads shellfish production in Latin America, harvesting 431,300 t in 2021 (SUBPESCA, 2023). Building on this strong base, Chile has potential to expand and diversify its aquaculture production. One promising species is the Pacific Oyster, *Magallana gigas*, introduced to Chile in the 1970s. Its production peaked in 2000, reaching approximately 7000 tons. However, the detection and persistence of Norovirus, a causative agent of gastroenteritis in humans and associated with oysters cultivated in Chile, led to export bans in 2006 (Abarca et al., 2025). Since then, both private and governmental efforts have aimed to revive oyster production, supporting the development of large- and small-scale aquaculture (Abarca et al., 2025). From 2013, farming of *M. gigas* has been carried out mainly in the Coquimbo (mainly at Tongoy Bay) and Los Lagos regions (*i.e.*, northern and southern Chile, respectively) with a peak of 442 tons in 2019 followed by a steep decline reaching 16 tons by 2023 (Servicio Nacional de Pesca y Acuicultura, 2023), highlighting the underdeveloped state of this industry in Chile.

Ensuring the sustainable development of *M. gigas* production in new farming regions requires not only assessing the technical aspects of bivalve rearing – an area where Chile already has experience. It also demands proactive management of infectious diseases, phenomenon that inevitably accompanies and hinders the development of aquaculture. As highlighted in the literature, biosecurity in aquaculture involves preventive strategies to reduce the socioeconomic burden of disease in farming sites (Aly and Fathi, 2024; Fox et al., 2020; Subasinghe et al., 2023). So far, no major mortality events have been registered in oyster farms in Chile, and key primary pathogens of oyster such as *Ostreid herpesvirus 1* (OsHV-1) and *Vibrio aestuarianus* (D'egremont et al., 2021) remains undetected.

However, vibrios – naturally associated with oysters as part of their microbiota – can act as opportunistic pathogens under certain conditions of biotic and abiotic stresses that impact oyster health (King et al., 2019; de Lorgeril et al., 2018; Oyanedel et al., 2023). Under normal conditions, healthy oysters limit *Vibrio* proliferation through their immune defenses (Bruto et al., 2017). Disturbance of this immune homeostasis can open the door to the pathogenesis, where vibrios equipped with diverse virulence factors are able to lead to oyster mortality.

Several virulence factors have been identified and shown to play key roles in oyster vibriosis, including the protein r5.7 found in *V. splendidus*, Type VI secretion systems (T6SS)—which are molecular syringes that inject toxic effectors into eukaryotic host cells, protist predators like amoebas, or prokaryotic competitors (Coulthurst, 2013) —and multifunctional auto-processing repeats-in-toxin (MARTX) toxins, which are large modular toxins secreted through an atypical Type I secretion system (Bruto et al., 2018; Oyanedel et al., 2020). Horizontal gene transfer occurs at high frequency among vibrios (Roux and Blokesch, 2018), facilitating the dissemination of virulence factors that cause tissue damage and are exploited for nutrition. These traits, which play a key role in the outcome of host-pathogen interactions can be positively selected, particularly in high-density host populations, such as farmed oysters (Bruto et al., 2017). Therefore, the epidemiologically silent presence of virulence factors within oysters should not be considered as an absence of risk and discourage the efforts to actively monitor their occurrence, frequency and to estimate their potential contribution to pathogenesis.

In previous work, we characterized the population structure of *Vibrio* communities isolated from healthy oysters, from a farm at Tongoy bay, along with their virulence potential and biochemical activities associated with specific virulence factors (Oyanedel et al., 2022). In the present study, by assessing the hemocyte-*Vibrio in-vitro* interactions and comparative genomics between these *Vibrio* strains, we identified known risk-associated virulence factors present in the most virulent and cytotoxic strains in our collection. We further estimated the seasonal frequency of these factors in *Vibrio* isolated in the field, providing new insights into their potential contribution to pathogenesis of farmed oyster.

## 2. Materials and methods

### 2.1. *Vibrio* strains, isolates and culture conditions

Twenty previously isolated and taxonomically assigned *Vibrio* were used in this study for cytotoxicity assays, fluorescence microscopy and genome sequencing. These included: *V. atlanticus* TO3\_18, *V. atlanticus* TO6\_8, *V. cyclitrophicus* TO1\_2, *V. cyclitrophicus* TO2\_1, *V. cyclitrophicus* TO4\_2, *V. cyclitrophicus* TO4\_17, *V. fortis* TO1\_18, *V. lentus* TO6\_3, *V. lentus* TO6\_11, *V. splendidus* TO3\_6, *V. splendidus* TO5\_9, *V. splendidus* TO10\_14, *V. splendidus* TO2\_12, *V. splendidus* TO2\_15, *V. splendidus* TO10\_4, *V. tapetis* TO3\_16, *V. tapetis* TO5\_8, *V. toranzoniae* TO1\_1, *V. toranzoniae* TO4\_1 and *V. toranzoniae* TO4\_3 (Oyanedel

et al., 2022). Additionally, another *Vibrio* collection was obtained from a seasonal isolation over a 12-month period to estimate the frequency of virulence factors in oyster in the field.

All *Vibrio* strains, and isolates were cultured at 18 °C in Trypticase Soy Broth (TSB) (Difco) supplemented with 0.5 M NaCl (TSBS) and Trypticase Soy Agar (TSA) (Difco) supplemented with 0.5 M NaCl (TSAS). For *Vibrio* strains carrying the pMRB-P<sub>lac</sub>GFP replicative plasmid (Roux et al., 2011) the culture media were supplemented with 10 µg mL<sup>-1</sup> of Chloramphenicol (Cm). *Escherichia coli* β2163 DAP<sup>-</sup> carrying the pMRB-P<sub>lac</sub>GFP, for conjugation assays with *Vibrio*, was cultured at 37 °C in TSA or TSB supplemented with Diaminopimelic acid (DAP) 300 µM (Oyanedel et al., 2022).

## 2.2. Animals

All experiments that included oysters complied with regulations on the protection of animals used for experimental and scientific purposes according to (ASAB, 2020) and was approved by Scientific Ethical Committee of the Universidad Catolica del Norte (Approval number: CEC-UCN N°14).

For the cytotoxicity assays, adult oysters ( $n = 30$ ) were obtained from a local farm in Tongoy Bay, Coquimbo region. Individuals were selected based on rapid valve closure upon tactile stimulation and their ability to remain tightly closed when handled. After selection, oysters were transported to the laboratory of Genetics and molecular Immunology at the Pontificia Universidad Catolica de Valparaíso, with no mortalities occurring during transportation. Prior to experimentation, oysters were acclimated for 3 days in a 100-L recirculating system containing 60 L of filtered seawater at 15 °C, with constant aeration. Each day, 10 % of the seawater volume was replaced. During acclimation, no mortalities were recorded. In addition, hemocytes consistently showed high viability, normal morphology, and rapid adhesion under culture conditions in all cytotoxicity assays that were made in the following 4 days, which was interpreted as evidence of the good health status of the animals.

For the seasonal *Vibrio* isolation, oysters were transfer from the aquaculture facilities of the Universidad Catolica del Norte (UCN) at Tongoy bay to the Central Marine Culture Laboratory of the UCN (LCCM-UCN) at Coquimbo city and immediately process for *Vibrio* isolation.

## 2.3. Seasonal *Vibrio* isolation

To estimate the frequency of virulence factors in the field, a seasonal *Vibrio* isolation was performed in autumn (May 2022), winter (August 2022), spring (December 2022), and summer (March 2023). At each season, ten oysters were individually homogenized together in 3 g of sterile seawater per g of oyster tissue. We performed 10-fold dilutions of the 10 tissue homogenates and plated 100 µL of the 10<sup>-1</sup> and 10<sup>-2</sup> dilution on TCBS plates, in duplicate. Around ten colonies per sample were randomly selected and re-stroked twice in TSA 2 % NaCl, before producing 10 % glycerol stocks and storing them at - 80 °C to produce a collection of 310 isolates.

## 2.4. Cytotoxicity assay

To complement our previously reported virulence data and gain further insight into the pathogenic potential of these strains, we assessed cytotoxicity, which often correlates with virulence *in vivo* (Rubio et al., 2019), in our 20 *Vibrio* strains.

Hemolymph was extracted from the abductor muscle of adult oysters using a 25-gauge syringe, with syringes and collection tubes maintained on ice to prevent hemocyte aggregation (Piel et al., 2020; Oyanedel et al., 2023; Silva Freire et al., 2023). Hemolymph from 3 to 4 oysters was pooled in a 50 mL polypropylene tube kept on ice. Hemocytes were typically extracted and seeded within 20 min of collection, a timeframe that consistently yielded high-quality monolayers, with hemocytes adhering and beginning to spread within 20 min after seeding. The number of hemocyte in the pooled hemolymph was estimated using a manual hemocytometer and an appropriate volume of hemolymph was placed in 96 well-plates to seed 2 × 10<sup>5</sup> hemocytes/well.

The remaining hemolymph was centrifuged to remove hemocytes and the resulting plasma filtered through 0,22 µm filters. Hemocytes were allowed to adhere to the bottom of the wells for 1 h at 18 °C. After adhesion, plasma was removed from the wells and replaced by 200 µL of 5 µg/µL Sytox Green (Molecular Probes) prepared in sterile seawater.

In parallel, stationary 18 h cultures of the 20 *Vibrio* strains, inoculated the day before from fresh cultures in TSAS plates, were centrifuged, resuspended in filtered plasma and incubated at 18 °C for 1 h. Since plasma is replaced by seawater in the assay wells, this pre- incubation, allows *Vibrio* opsonization with plasma factors facilitating subsequent interactions with hemocytes. *Vibrio* suspensions were adjusted to an OD<sub>600</sub> of 1 (10<sup>9</sup> cfu/mL) and added to the wells at a multiplicity of infection (MOI) of 50:1. *Vibrio* CFU concentration was confirmed to be at 10<sup>9</sup> by conventional dilution plating. SYTOX™ Green fluorescence was monitored (λ<sub>ex</sub> 480 nm/λ<sub>em</sub>550nm) for 18 h using a TECAN microplate reader. Conditions included in the cytotoxicity assay were (i) Control hemocytes without *Vibrio*, (ii) hemocytes and *Vibrio* strains, and (iii) hemocytes in 0,1 % Triton X-100 to obtain a SYTOX™ Green

signal associated to a 100 % hemocyte cytolysis. This value of maximum cytolysis was used on each assay to calculate the percentage of hemocyte lysis. Three technical replicates (wells) were used per condition. This experiment was repeated four times on different days. Data presented correspond to the maximum cell lysis produced by each strain.

## 2.5. Whole genome sequencing and phylogenomic analysis

We selected a subset of eight *Vibrio* strains representing distinct combinations of *in vivo* virulence and *in vitro* cytotoxicity (Supplementary Table 2). This strategy aimed to maximize contrast among phenotypic profiles, thereby facilitating the identification of genomic features associated with pathogenic potential. The selected strains included virulent and cytotoxic strains, non-virulent and non-cytotoxic ones, as well as intermediate or discordant phenotypes, enabling a more comprehensive analysis of the molecular basis of virulence.

Genomic DNA was purified from liquid cultures of the eight selected *Vibrio* strains with the Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instruction. DNA integrity was verified through agarose electrophoresis and concentration estimated through a Qubit instrument (Thermo Scientific).

The whole-genome sequencing (WGS) and assembly of the gDNA was performed by Plasmidsaurus (Oregon, United States), using long-read sequencing from Oxford Nanopore Technologies (ONT). The process included the following steps: construction of an amplification-free long-read sequencing library, using v14 library prep chemistry; sequencing the library primer-free, using R10.4.1 flow cells. Low-quality reads were filtered using FilTlong v0.2.1 (Wick, 2018), and the assembly of the genomes was performed using miniasm v0.3 (Li et al., 2019) and Flye v2.9.1 (Kolmogorov et al., 2019). The assembly was polished using Medaka v1.8.0. The genomes were annotated using Bakta v1.6.1 (Schwengers et al., 2021). The contigs were analyzed with Bandage v0.8.1 (Wick et al., 2015). Genome completeness and contamination were verified using CheckM v1.2.2. (Parks et al., 2015). *Vibrio* strains were assigned to a species using the Similar Genome Finder Service available in the Bacterial and Viral Bioinformatics Resource Center (BV-BRC). Briefly, genome distance (D) was computed using Mash/MinHash between each genome and the public genomes available in the BV-BRC database with parameters set as follows: Max hits: 50; P-Value Threshold:1; Distance: 1; Organism type: bacterial and archaeal genomes; Scope: all public genomes. Strains were assigned to the closest species. A rooted phylogenetic tree was inferred using the OrthoFinder software (v2.5.4) (<https://github.com/davidemms/OrthoFinder>) with the genomes of our strains, the closest strains based on genomic distance, and a set of reference genomes of the assigned species. OrthoFinder (options: -f -M msa) was used to define orthogroups. Then, single copy orthologs were concatenated and aligned with MAFFT (v7.525) and maximum likelihood phylogenetic tree was inferred from the multiple sequence alignment using FastTree (v2.1.11), with *V. breoganii* FF50 used as outgroup. The tree was plotted in the iTOL platform.

## 2.6. Prediction of macromolecular systems and virulence factors

Genomes were uploaded to the Microbial Genome Annotation & Analysis Platform (MAGE) (<https://mage.genoscope.cns.fr/microscope/home/index.php>). We used the MAGE platform built-in MacSyFinder tool to obtain the predicted macromolecular systems on each genome. For the prediction of virulence factor, we use the VFAnalyzer tool available in the VFDB Virulence factor database (<https://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFAnalyzerhttps://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi>).

The VFAnalyzer pipeline was used choosing Proteins of ALL predicted CDSs in FASTA format as the upload file type. Results from MacSyFinder and VFAnalyzer were download and downstream analyses were conducted in R environment (v4.1.2).

## 2.7. GFP labelling of *Vibrios*

To visualize bacterial–host cell interactions, the eight genome-sequenced *Vibrio* strains were transformed with the pMRB plasmid containing the green fluorescent protein (GFP) gene, which is known to be stable in *Vibrio spp.* (Le Roux et al., 2011). This construct allows for constitutive GFP expression driven by a pLac promoter under chloramphenicol selection, enabling fluorescence-based detection of *Vibrio* cells during microscopy analyses of their interaction with oyster hemocytes. Briefly, one colony from stationary cultures of *Vibrio* and *E. coli* B3914 (auxotroph for Diaminopimelic acid) carrying pMRB-pLacGFP cultured in their respective agar were mixed into TSAS + Diaminopimelic acid (DAP) and kept at 20 °C for 24 h. Next, a loopful of the bacterial mixture was taken with an inoculation loop and resuspended into TSBS. The bacterial suspension was serially diluted in ten-fold steps and 100 µL of the last three dilutions were plated into TSBS + Cm at 10 µg mL<sup>-1</sup> and without DAP to select transformant vibrios and counter select DAP- auxotrophic *E. coli* B3914, respectively. Three transformant colonies per strain were

re-streaked in TSBS + Cm at  $10 \mu\text{g mL}^{-1}$ . All strains were successfully transformed, and GFP-labeled *Vibrios* were stored in TSBS + Cm at  $10 \mu\text{g mL}^{-1}$  + 15 % glycerol at  $-80^\circ\text{C}$ . Fluorescence was confirmed in a UV transilluminator.

## 2.8. Fluorescence microscopy

Hemolymph was pooled from 3 to 4 oysters and hemocytes were seeded onto glass coverslips in a 24-well plate to obtain monolayers of  $5 \times 10^5$  cells per well. Adherent hemocytes were exposed to GFP- expressing *Vibrio*, that had been washed with sterile seawater and opsonized in filtered oyster plasma for 1 h. The eight GFP-labeled *Vibrio* strains were added at a multiplicity of infection of 50:1. After a 3 h incubation, the cell monolayers (coverslips placed at the bottom of the wells) were fixed with 4 % paraformaldehyde in PBS (pH 7.7) for 15 min. Coverslips were then washed thrice in PBS and permeabilized with 0.5 % Triton x 100 for 10 min at room temperature. Coverslips then were blocked with 1 % bovine serum albumin (BSA) in PBS for 1 h at room temperature. Actin was visualized with Phalloidin 568 (Thermo Scientific) and TO-PRO iodide<sup>®</sup> (Thermo Scientific) was used as nuclear contrast. The coverslips were then mounted in Heathshield mounting medium (Vector). Imaging was performed using a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems) with a Leica  $40 \times 1.25$  Oil HCX APO CS lens (Leica Microsystems).

## 2.9. PCR-based detection of virulence genes

We design primers to amplify a conserved region of the *hcp* and *rtxA* genes. Primers used are listed in Supplementary Table 1. For T6SS detection, primers were designed based on the *hcp* sequences of strains TO6\_11 ( $V^+/C^-$ ), TO5\_9 ( $V^+/C^+$ ), and TO3\_6 ( $V^+/C^-$ ), which harbor a highly conserved T6SS cluster (Supplementary Fig. 2) and are associated to phenotypes of our interest in terms of virulence and/or cytotoxicity.

For MARTX detection, primers targeted the *rtxA* gene sequence from strain TO6\_11. Primer validation was performed using genomic DNA from the eight sequenced strains. Total DNA was quantified by nanodrop and normalized to  $50 \text{ ng}/\mu\text{L}$ . Each 25  $\mu\text{L}$  reaction mixture contained 1 $\times$  PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP, 0.4  $\mu\text{M}$  of each primer, 1 U of Taq DNA polymerase (Thermo Fisher Scientific), and 50 ng of genomic DNA. PCR amplifications were carried out in a thermal cycler under the following conditions: initial denaturation at  $95^\circ\text{C}$  for 5 min; followed by 35 cycles of denaturation at  $95^\circ\text{C}$  for 30 s, annealing at  $55^\circ\text{C}$  for 30 s, and extension at  $72^\circ\text{C}$  for 1 min; with a final extension step at  $72^\circ\text{C}$  for 7 min. PCR products were resolved by electrophoresis on a 1.5 % agarose gel stained with GelRed (Thermo Scientific) and visualized under UV light in a ChemiDoc Imaging System (Bio-Rad). Amplification was observed only in strains carrying the corresponding target gene. The specificity of amplification was verified by agarose gel electrophoresis, which confirmed bands of the expected size exclusively in the positive strains (Supplementary Fig. 1).

For seasonal *Vibrio*, template DNA was obtained by boiling one isolated colony from each *Vibrio* for five minutes in 100  $\mu\text{L}$  of molecular- grade water. After boiling, samples were centrifuged at  $10.000 \times g$  for 5 min and the supernatant recovered and stored at  $-20^\circ\text{C}$ . PCR reactions were then performed under the same conditions described above.

## 3. Results

### 3.1. Virulent vibrio strains display cytotoxic activity against hemocytes in vitro

We screened our panel of 20 *Vibrio* strains previously categorized as virulent or non-virulent (Oyanedel et al., 2022), to estimate their cytotoxicity against hemocytes as another proxy for their potential pathogenicity against oysters.

As previously observed with virulence, cytotoxicity was highly variable among strains ranging from  $1.76 \pm 1.07 \%$  to  $41.48 \pm 12.98 \%$  of cell lysis produced by *V. atlanticus*.

TO3\_18 and *V. lentus* TO6\_3, respectively (Fig. 1). Three strains produced a degree of cell lysis significantly different than the control group without bacteria: *V. lentus* TO6\_3 ( $41.48 \pm 12.98 \%$ ,  $p < 0.0001$ ), *V. splendidus* TO2\_15 ( $33.76 \pm 10.04 \%$ ,  $p = 0.00368$ ) and *V. splendidus* TO5\_9 ( $39.98 \pm 21.52 \%$ ,  $p = 0.00019$ ) (Fig. 1). A positive correlation was observed between virulence and cytotoxicity (Pearson's correlation coefficient,  $r = 0.482$ ,  $p = 0.0315$ ). The strains *V. splendidus* TO2\_15, *V. splendidus* TO5\_9, previously characterized as virulent, also produced a significant degree of cytolysis in our assay. Despite the positive correlation between virulence and cytotoxicity at the global level, certain strains deviate from this trend. For instance, *V. lentus* TO6\_3 displayed significant cytotoxicity despite been previously categorized as non- virulent, and the most virulent strain *V. lentus* TO6\_11 produced a non-significant 23.45 % of cytolysis ( $p$  value = 0.168).

Strains that induced significantly higher hemocyte lysis compared to the control condition were classified as cytotoxic, while the remaining strains were considered non-cytotoxic. The virulence and cytotoxicity status of the 20 strains is summarized in the Supplementary Table 2.

### 3.2. Whole genome sequencing

Eight draft genomes were obtained through Oxford Nanopore sequencing. Assembly quality was confirmed using standard statistics, including genome size, coverage, contig number, N50 values and completeness (Supplementary Table 3). The initial taxonomic assignment of assembled contigs, performed by the sequencing platform using Mash (v2.3), indicated that some contigs shared high identity with plasmid sequences (Supplementary Table 4).

Based on genome-genome identity analyses, we confirmed the *hsp60*-based species assignment of *V. tapetis* TO3\_16, *V. cyclitrophicus* TO1\_2, *V. splendidus* TO10\_14, *V. lentus* TO6\_3 and *V. lentus* TO6\_1. Additionally, we reassigned *V. splendidus* TO5\_9 and *V. splendidus* TO3\_6 as *V. lentus*. A phylogenomic analysis including reference *Vibrio* genomes and the closest strains to our strains further supported these classifications, placing our strains in distinct clades alongside the reference strains of their respective species (Fig. 2).

### 3.3. *Vibrio* strains harbor several macromolecular systems and virulence-associated factors

To explore potential mechanisms underlying the observed phenotypes, we analyzed the genomes of the selected strains for virulence-associated determinants. Several macromolecular machineries in vibrios contribute to their virulence by aiding in mobility, adhesion, and through the secretion of toxic effectors. The MacSyFinder analysis revealed the distribution of such macromolecular machineries across the eight *Vibrio* genomes (Fig. 3A). Systems required for environmental persistence and host association, such as the flagellar apparatus, Type I and Type II secretion systems (T1SS, T2SS), tight-adherence (Tad) machinery, and type IVa pili (T4aP), were ubiquitous across strains. In contrast, elements involved in horizontal gene transfer and contact-dependent functions varied markedly: conjugative modules (dCONJ\_typeG), mobilization systems (MOB), and a Type IV secretion system (T4SS\_typeF) occurred only in a subset of genomes (Fig. 3A).

We also identified components of the Type V (T5cSS) and Type VI secretion systems (T6SS). Type V secretion are single-component secretion systems consisting of only one polypeptide chain with a  $\beta$ -barrel translocator domain in the membrane, and an extracellular passenger or effector region (Meuskens et al., 2019). Three T5cSS trimeric autotransporters were present in the genome of *V. splendidus* TO5\_9, including a YadA-like adhesin and two exported proteins of unknown function. Additionally, single YadA-like C-terminal membrane anchor domain-containing protein were found in *V. splendidus* TO3\_6 and *V. lentus* TO10\_14. While these proteins are often associated with virulence, their functional role in *Vibrio* remains unclear. At the other end of secretion complexity, we found one highly conserved T6SS module shared by *V. lentus* TO6\_11, *V. lentus* TO5\_9 and *V. splendidus* TO3\_6. *V. lentus* TO5\_9 carried a second, strain-specific T6SS module, while *V. tapetis* TO3\_16 possessed three unique T6SS modules not found in any other strain (Supplementary Fig. 2).

Since no clear correlation was observed between cytotoxicity and the presence of T6SS and considering that these systems can target either eukaryotic or prokaryotic cells, we further search for putative effectors that could better define their potential targets. Using the online tool Secret6 (<https://bioinfo-mml.sjtu.edu.cn/SecReT6/index.php>), several putative T6SS effectors were identified. Manual curation of the candidate sequences, together with the proteins already identified, revealed the presence of PAAR domain-containing proteins in *V. lentus* TO6\_11, TO3\_6, and TO5\_9; a DUF4150 domain-containing protein in *V. lentus* TO5\_9; and VgrG and Hcp proteins in all strains. No toxin-associated domains were detected through InterProScan in these structural proteins that could help to define the cellular target of these T6SSs.

Also, although not direct effectors, several cold-shock proteins with reported virulence-regulating activity (Eshwar et al., 2017) were also identified (Supplementary Table 5).

Complementing the identification of macromolecular systems, we also compared our *Vibrio* genomes against a curated database of annotated virulence factors from pathogenic bacteria. This analysis revealed a total of 35 distinct virulence factors grouped into 21 functional classes (VfClass) across the eight strains genomes (Fig. 3B). Within these classes, iron uptake was the leading category, with six associated different virulence factors, followed by adherence and toxin with four each and antiphagocytosis, endotoxin and quorum sensing with two each. Several virulence factors were conserved across all strains, including Type IV pilus, Capsular polysaccharide, Capsule (Acinetobacter), Heme receptors, Periplasmic binding protein-dependent ABC transport systems, Autoinducer-2, Cholerae autoinducer-1, Two-component system (Acinetobacter), and Thermolabile hemolysin.

When exploring the exclusive virulence factors of each strain, we found that only the virulent *V. lentus* TO6\_11 harbors a complete Multifunctional Autoprocessing Repeats-in-Toxin (MARTX) cluster (*rtxACHBDE*) (Supplementary Fig. 3).

Given the mosaic nature of the MARTX holotoxin, we compared the domain architecture of the MARTX of *V. lentus* TO6\_11, herein MARTX<sub>TO6\_11</sub>, with those from other bacteria. Our analysis showed that this toxin corresponds to an F-type MARTX (Woida et al., 2022), with a module composition found in *V. splendidus* strains with virulence potential towards oysters (Bruto et al., 2018). Specifically, it showed a 94,34 % protein identity with the MARTX of *V. splendidus* 5S-101 and shared the same effector domain composition and organization (Fig. 4.)

MARTX<sub>TO6\_11</sub> features conserved amino- and carboxyl-termini domains. The variable region contains an actin crosslinking domain (ACD), an alpha/beta hydrolase (ABH), a Ras/Rap1-specific endopeptidase (RRSP), a cysteine protease domain (CPD), required for the cleavage of the toxin precursors, and two other cysteine protease effectors domains known as Make Caterpillar Floppy domain (MCF) (Fig. 4).

### 3.4. *V. splendidus* TO6\_11 induces cell rounding on hemocytes in vitro

The cytopathic effect of the 8 *Vibrio* strains on hemocytes was determined using fluorescence microscopy.

The MARTX cluster was found exclusive of TO6\_11. Although this strain did not show significant high cytotoxicity (23,45 %,  $p = 0.16752$ ), we investigated whether the presence of the MARTX toxin was associated with morphological changes on oyster hemocytes. After 3 h of determined using fluorescence microscopy.

The MARTX cluster was found exclusive of TO6\_11. Although this strain did not show significant high cytotoxicity (23,45 %,  $p = 0.16752$ ), we investigated whether the presence of the MARTX toxin was associated with morphological changes on oyster hemocytes. After 3 h of contact with hemocytes, TO6\_11 caused a marked alteration in hemocyte morphology (Fig. 5). Hemocytes, which normally adhere and spread in a fibroblast-like morphology, appeared completely rounded when exposed to TO6\_11. No other strain produced this effect on hemocytes morphology, not even *V. lentus* TO6\_3 and *V. splendidus* TO5\_9 despite their ability to significantly lyse hemocytes in vitro (Fig. 1).

### 3.5. Seasonal variation in the frequency of T6SS and MARTX toxin genes

Seasonal screening revealed striking differences in the prevalence of virulence factors. Isolates carrying the T6SS (*hcp* gene) showed pronounced seasonal fluctuation, reaching a peak frequency of 70.6 % in winter, a 7.1-fold increase compared to autumn, before declining to 10.3 % in summer (Fig. 6). In contrast, the MARTX toxin (*rtxA* gene) remained a consistently low frequencies (1.0–2.4 %) throughout the year, showing no significant seasonal pattern. These results suggest that, so far, MARTX represents a stable but minor component of the *Vibrio* virulence repertoire in *M. gigas* farming sites in Chile.

## 4. Discussion

*Vibrios* are part of the natural microbiota of oysters and are commonly detected in their tissues (Soree et al., 2022). However, the oyster's internal environment generally poses significant challenges to *Vibrio* proliferation due to the arsenal of cellular and molecular antibacterial defenses provided by innate immunity (Zhang et al., 2024). When this immune homeostasis is compromised by biotic (de Lorgeril et al., 2018) or abiotic stressors (Scanes et al., 2025), opportunistic pathogens, such as *Vibrios* equipped with virulence factors, proliferate not only causing harm to the host's tissues but also disrupting the resident microbial community, ultimately leading to oyster death (Lokmer and Mathias Wegner, 2015). Selective pressures maintaining this virulence factors within the *Vibrio* community outside disease could come from the highly dynamic character of the association between *Vibrios* and oysters. These bacteria are constantly exposed to other environmental organisms, such as protozoan grazers. This can exert selective pressure to maintain secretion systems and toxins that enhance environmental survival, but which also function as virulence determinants during oyster infection (Erken et al., 2013; Matz and Kjelleberg, 2005).

By experimentally introducing *Vibrio* strains directly into the oyster's hemolymph, bypassing several of these defense barriers, it becomes evident that while some strains can trigger mortality, others appear harmless even under these artificially permissive conditions (Oyanedel et al., 2022).

In the present study, we further characterized selected *Vibrio* strain candidates to identify virulence determinants. These determinants may play critical roles in disease emergence under future scenarios of intensified aquaculture or environmental stressors. Specifically, we focused on 20 *Vibrio* strains that had been previously taxonomically assigned and defined as virulent or non-virulent, to explore the molecular basis underlying their pathogenic potential (Oyanedel et al., 2022). Overall, we observed a positive correlation between virulence and cytotoxicity across the analyzed strains, supporting cytotoxicity as a useful indicator

of pathogenicity. However, certain strains exhibited divergent profiles. Notably, *V. lentus* TO6\_11 categorized as virulent displayed a non-significant level of cytotoxicity (23.45 %,  $p = 0.168$ ), whereas the non-virulent *V. lentus* TO6\_3 induced significant cytotoxicity (41.4 %,  $p < 0.0001$ ). These differences likely arise from the nature of the assays used. *In vivo* injection experiments and *in vitro* hemocyte cytotoxicity tests do not attempt to reproduce natural infection routes but rather serve as standardized approaches to reveal the capacity of certain strains to cause oyster mortality and/or hemocyte lysis. While useful as preliminary screening tools, these two methods place host and bacteria in substantially different contexts and therefore perfect concordance between the two approaches should not necessarily be expected.

Successful infection involves more than simply impairing hemocyte function. *Vibrios* must overcome the host's humoral immune factors, resist antimicrobial peptides and reactive oxygen species, acquire iron *via* siderophore systems, and establish adhesion and colonization on host tissues (Destoumieux-Garzon et al., 2024; Destoumieux-Garzon et al., 2020; Destoumieux-Garzon et al., 2014). Our comparative genomic analysis revealed that *V. lentus* TO6\_3 lacks the Mannose-Sensitive Haemagglutinin (MSHA) pilus, a structure to mediate adhesion to mussel hemocytes *in vitro* (Zampini et al., 2003). We can hypothesize that the absence of this or other adhesion-related factors could limit this strain ability to colonize host tissues, thus reducing its virulence *in vivo* despite the cytotoxic activity displayed under *in vitro* conditions.

While *in vitro* assays cannot fully replicate the complexity of host- microbe interactions *in vivo* and should be interpreted with this limitation in mind, our findings support the value of cytotoxicity assays as a preliminary tool to assess the pathogenic potential of environmental and host-associated strains, thereby guiding future studies under more ecologically realistic conditions.

Functional genome analyses revealed the presence of several virulence-associated systems, including secretion systems, adhesion structures, and iron acquisition pathways. The presence of these determinants in strains associated with healthy oysters is likely explained by their multifunctional roles beyond virulence. Many so-called virulence factors contribute to ecological fitness, such as nutrient acquisition, resistance to antimicrobial compounds, or defense against protozoan grazers. Interactions with amoebae and other microbial predators have been proposed as important selective forces maintaining secretion systems and toxins that, while primarily enhancing survival in the environment, can also act as virulence determinants during infection (Erken et al., 2013; Matz and Kjelleberg, 2005).

Among these identified virulence factors, the Type VI Secretion System (T6SS) showed the most variable distribution. Although the contribution of some T6SS to cytotoxicity towards hemocytes has been functionally validated (Piel et al., 2020; Rubio et al., 2019), we did not find a strong association of their presence with the cytotoxicity profile of our strains.

Nonetheless, several putative T6SS effectors were identified across the studied *Vibrio* strains, such as VgrG, Hcp, and PAAR domain-containing proteins. These proteins are known to play structural and functional roles in T6SS-mediated effector delivery and have also been associated with toxic activities in diverse hosts, including oysters (Cianfanelli et al., 2016; Jana and Salomon, 2019). The DUF4150 domain-containing protein shares structural similarity with PAAR domains and is predicted to function as a cargo or tip-associated effector in T6SS, but its role in oyster-pathogenicity requires further validation (Shneider et al., 2013). A more in-depth search for possible effectors associated to these T6SSs will be required for future studies.

Although not direct effectors, several cold-shock proteins with described roles in the regulation of virulence gene expression and host adaptation were also identified (Limthammahisorn et al., 2008). The identification of these virulence factors highlights the value of draft genome assemblies to obtain preliminary insights into the pathogenic potential of newly isolated strains (Ahmed et al., 2024; Mannion et al., 2025). Such information remains highly informative, even in the absence of fully closed genomes, and guides the prioritization of future functional and genomic studies.

The presence/absence profile of virulence factors evidenced that, although not significantly cytotoxic, only *V. lentus* TO6\_11 presented a MARTX toxin and displayed a particular cell rounding cytopathic effect on hemocytes when we assessed the *Vibrio*-hemocyte interaction by fluorescence microscopy.

The MARTX cluster encodes the MARTX toxin (*rtxA*), an uncharacterized protein (*rtxH*), a putative acyltransferase (*rtxC*) and a putative type-I secretion system (*rtxBDE*) required for MARTX holotoxin secretion (Satchell, 2015).

MARTX toxins are known to disrupt cellular processes through three different mechanisms. The MARTX holotoxin (*rtxA*) can lead to cell rounding by the inactivation of the Rho GTPases carried out by the RID domain, by the ACD-dependent covalent actin crosslinking of G-actin monomers (Kudryashov et al., 2008), and by the induction of the intrinsic apoptosis pathway and another, yet uncharacterized, caspase-independent apoptotic mechanism by the MCF toxic module (Kim, 2018). In mammalian cells, actin crosslinking by MARTX can also ultimately lead to apoptosis (Suarez et al., 2012). The induction of programmed cell death seems to be a main activity of the MARTX holotoxins and should be validated in hemocytes. The previously reported virulence of TO6\_11 contrasts with its non-significant cytotoxicity observed here. This suggests that the MARTX toxin may contribute to pathogenesis through an alternative, predominantly non-lytic effect, such as the cell-rounding phenotype observed, which would not be detected by assays, such as SYTOX™ Green, focused solely on membrane integrity (Grootjans et al., 2016). This provides a possible explanation for how strain TO6\_11 could exert virulence despite showing limited cytolytic activity *in vitro*.

According to (Woida and Satchell, 2020), in *V. cholerae*, the actin crosslinking effect of ACD alone elicits a strong proinflammatory response. However, the co-deliverance of RID and ABH silences the ACD-induced inflammation. Both, RID and ABH domains are required for the complete abolition of the global inflammatory response induced by ACD, although they independently silence signal transduction pathways before the host cytoskeletal damage is detected. Since MARTX\_TO6\_11 carries both ACD and ABH domains, it is plausible that this toxin not only disrupts hemocyte morphology but also suppresses host immune responses. This immunomodulatory activity could promote bacterial colonization while evading host detection, a strategy that may contribute to chronic persistence or opportunistic pathogenicity in oysters. Given that reduced immunogenicity has been linked to increased *Vibrio* colonization success in oysters (Oyanedel et al., 2020), validating the anti-inflammatory potential of MARTX\_TO6\_11 in oyster models represents an important future research direction.

While already supported by the literature, the functional validation of both MARTX and the different T6SS found is required for further characterization of our strains, this is especially important for the T6SS, as not all systems studied in *Vibrio* strains pathogenic to oyster contribute to their virulence (Rubio et al., 2019).

Understanding the distribution of specific virulence factors in *Vibrio* communities is critical for developing targeted diagnostic tools and risk assessments in aquaculture (Zhang et al., 2025). To this end, we monitored the seasonal prevalence of T6SS and MARTX genes in oyster-associated *Vibrio* populations over one year. Our results showed a pronounced seasonal fluctuation in T6SS frequency, peaking in winter at 70.6 %, a 7.1-fold increase compared to autumn, before declining sharply in summer. Seasonal variability of *Vibrio* communities is a well-documented phenomenon. Environmental parameters such as salinity and temperature are major drivers of *Vibrio* abundance, with higher values generally associated with increased bacterial densities (Takemura et al., 2014). In terms of community structure, this genus also displays species-specific responses to general seasonal changes (Wang et al., 2020) and particular environmental variables such as temperature (Wan et al., 2025).

In this context, winter seasons, characterized by lower temperatures, higher dissolved oxygen, and lower salinity has been associated with a decline in *Vibrio* abundance (Siboni et al., 2016), whereas Wang et al. (2020) further related this period to reduced *Vibrio* diversity linked to low temperature, decreased chlorophyll  $\alpha$ , and limited organic matter availability.

The observed increase in T6SS frequency could indirectly reflect reduced community diversity, resulting in the predominance of T6SS-positive strains that are selectively advantaged under winter conditions, potentially in a species-specific manner that remains to be demonstrated. Shifts in the oyster-associated microbiota have been recognized as indicators of disease onset in bivalves (Lasa et al., 2019; Li et al., 2019). In our case, we cannot conclude that this shift is necessarily associated with a higher risk of mortality; however, it represents an additional factor to consider in the context of the seasonal variations experienced by oysters in the field. Whether the presence of T6SS contribute to shape the observed pattern or is merely coincidental remains an open question that warrants further investigation.

In contrast, the frequency of MARTX toxin gene (*rtxA*) remained consistently rare across all seasons, with frequencies ranging from 1.0 % to 2.4 %, indicating that, at present, MARTX is not a dominant trait in the *Vibrio* populations associated with farmed oysters in northern Chile. Nevertheless, MARTX has been identified as a key virulence factor in oyster-pathogenic vibrios (Bruto et al., 2018). Establishing a baseline prevalence provides a valuable reference for future monitoring, for example through periodic PCR screening of T6SS/MARTX in sentinel samples, to track long-term patterns and variations associated to environmental parameters and abnormal mortality rates. Such monitoring would enable the detection of shifts in virulence factor distribution that could signal emerging disease risks in oyster farming areas such as Tongoy Bay.

## 5. Conclusions

This study demonstrates that *Magallana gigas* oysters from Tongoy Bay harbor *Vibrio* strains carrying major virulence factors, including T6SS and MARTX toxins, even in the absence of disease outbreaks. Phenotypic and genomic analyses revealed distinct strategies of interaction with hemocytes, ranging from lytic to non-lytic cytopathic effects. Seasonal screening showed a marked winter increase in T6SS-positive isolates, whereas MARTX toxins remained consistently infrequent. These findings highlight the complexity of *Vibrio*-oyster dynamics and the multiplicity of possible virulence strategies. Overall, our work establishes a baseline reference for virulence factor prevalence in northern Chile and supports the integration of microbiological indicators into aquaculture monitoring.

### CRediT authorship contribution statement

**Daniel Oyanedel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

**Felipe Navarro:** Methodology, Data curation.

**Katherina Brokordt:** Writing – review & editing, Validation, Supervision, Resources.

**Paulina Schmitt:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology.

### **Declaration of generative AI and AI-assisted technologies in the writing process**

During the preparation of this work the authors used chatGPT/ OpenAI in order to improve language. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

### **Funding**

This work was supported by the National Agency for Research and Development (ANID), Postdoctoral FONDECYT #3220530 grant. This work was carried out within the framework of the International Research Project EcoHealth4Sea funded by the CNRS. It received funding from CAPES-COFEUCUB (Sentinels project, Sv1064/25, N° 52623VH).

### **Declaration of competing interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

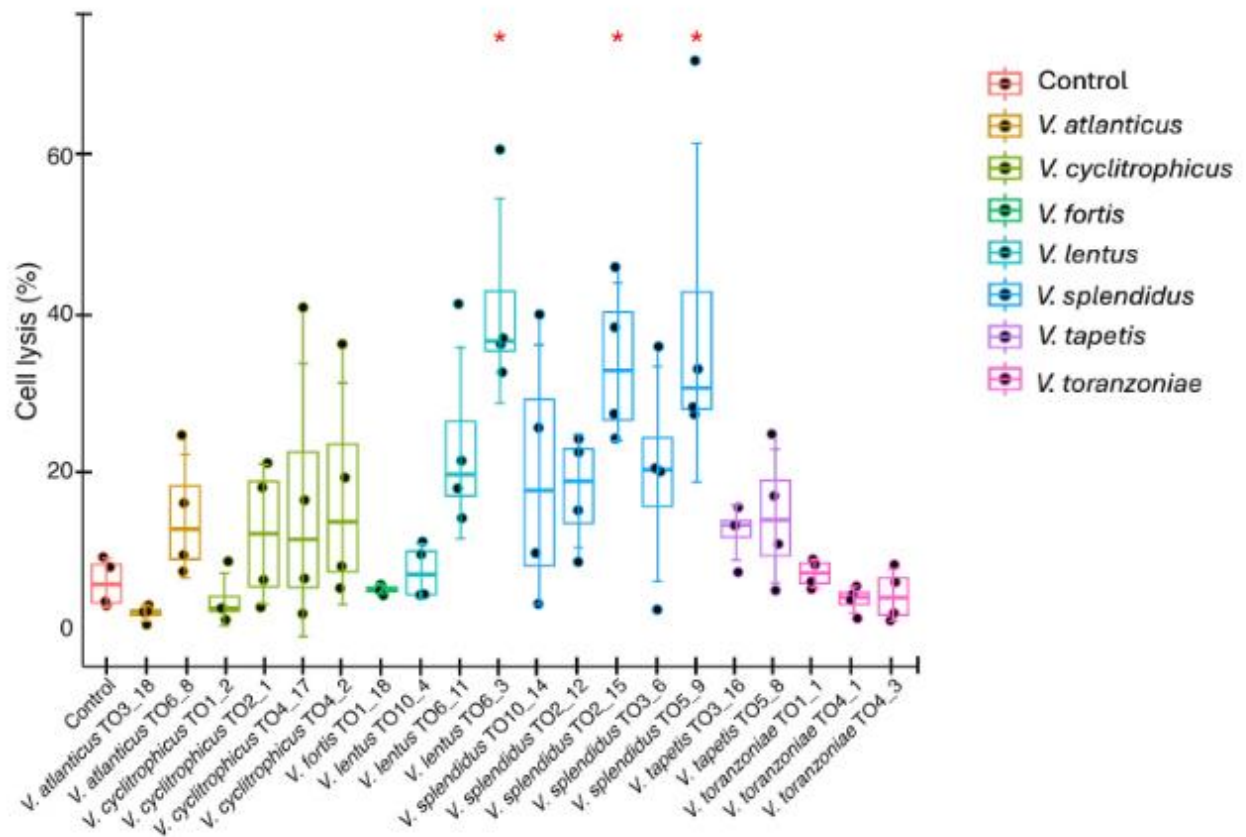
Daniel Oyanedel Trigo reports financial support was provided by National Agency for Research and Development. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### **Appendix A. Supplementary data**

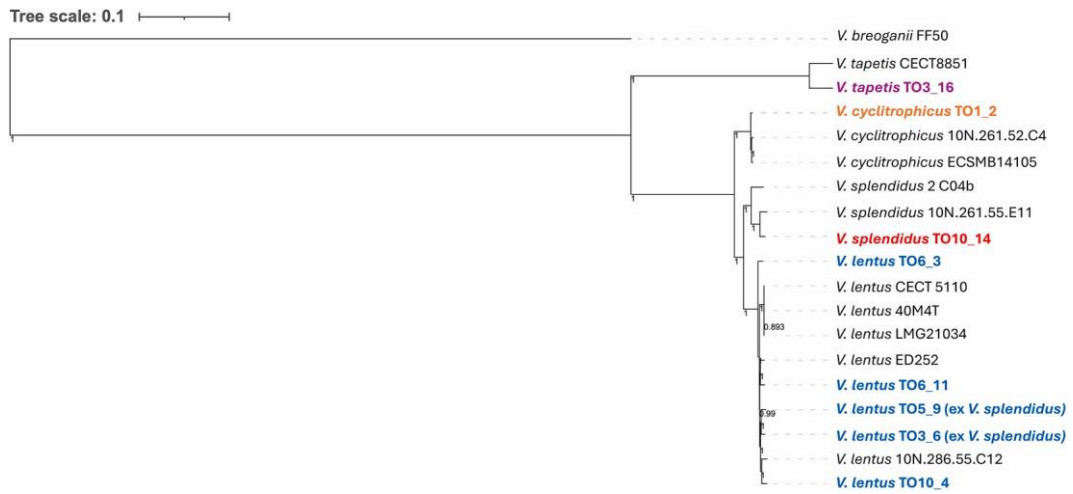
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2025.743391>.

### **Data availability**

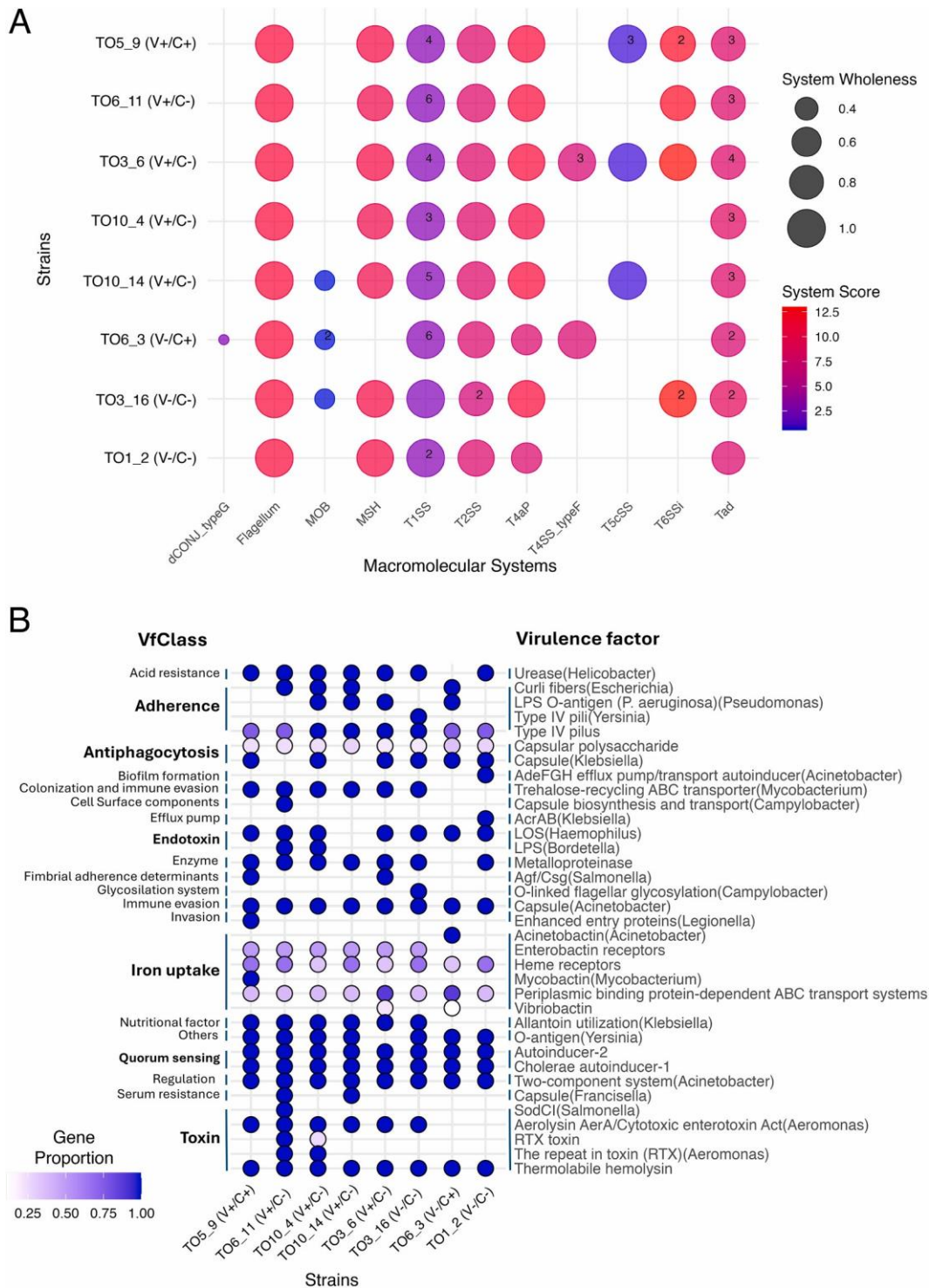
Data will be made available on request.



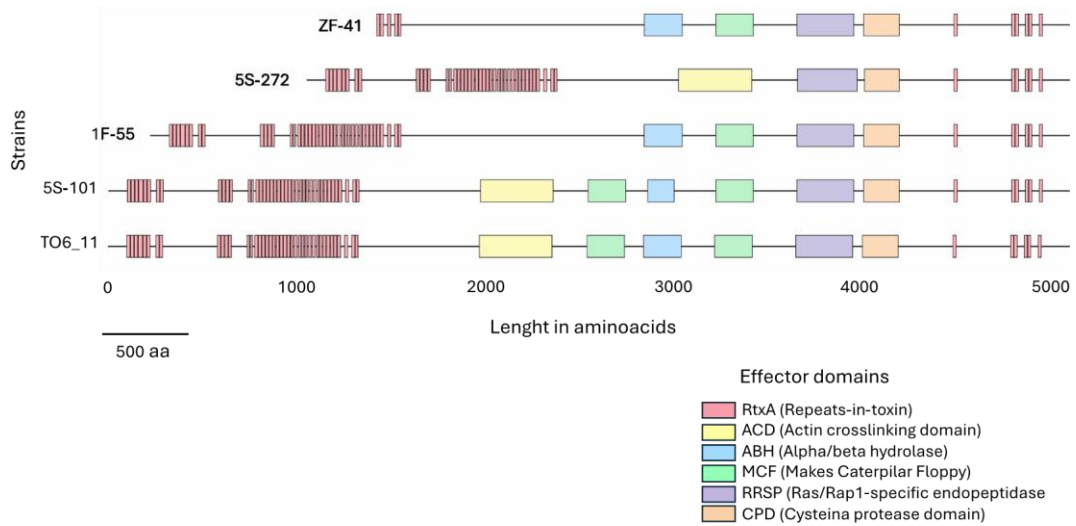
**Fig. 1.** *Vibrio* cytotoxicity against oyster hemocytes. Hemocyte lysis was detected using the DNA intercalating probe SYTOX™ Green. The graph shows the mean  $\pm$  SD of cell lysis values from four independent experiments, each with three technical replicates. Statistical differences in cell lysis were assessed using the Kruskal-Wallis test, followed by a Dunnett's post-test comparing each *Vibrio* strain to the control. P-values were adjusted for multiple comparisons using the Bonferroni correction. Significant differences ( $p \leq 0.05$ ) are indicated by an asterisk (\*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



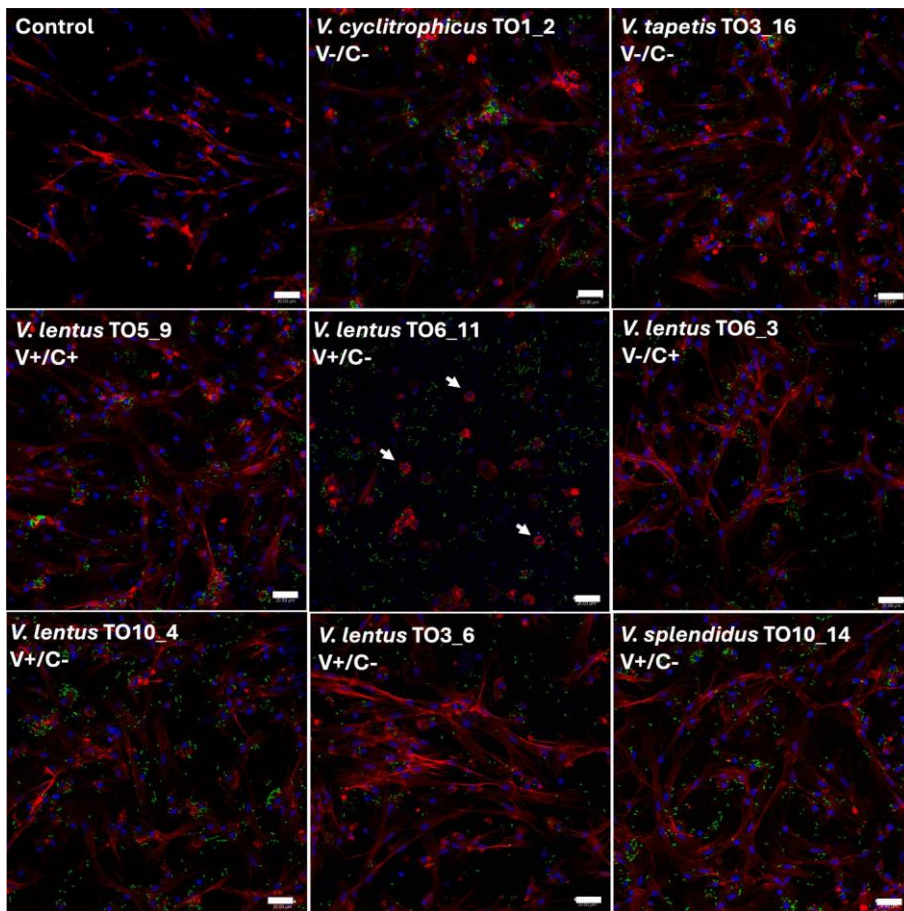
**Fig. 2.** Whole genome phylogenetic analysis of *Vibrio* strains. Phylogenomic tree inferred with FastTree based on multiple sequence alignments of single copy orthologs identified by Orthofinder. Branch local support values were calculated using the Shimodaira-Hasegawa test with 1000 resamples. Strains analyzed in this study are in bold and colored according to their assigned species.



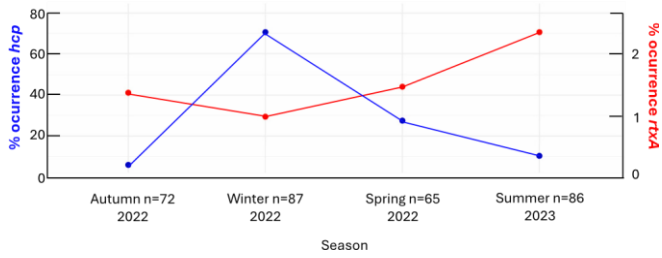
**Fig. 3.** Predicted macromolecular systems and annotated virulence factors in *Vibrio* strains. (A) Presence of macromolecular systems predicted with the MacSyFinder tool. Circle size represents system wholeness, and colour indicates the system score estimated by the MacSyFinder algorithm. The number inside the circles represent the number of systems identified for each type. (B) Predicted virulence factors using the VFAnalyzer tool. The colour scale represents the proportion of virulence-associated genes detected in each strain relative to the total number of genes defined for each virulence factor category. On the left are shown the functional classes of virulence factors, and on the right the specific virulence factors belonging to each class. Panels A and B include the virulence/cytotoxicity profile for each strain next to its name, with abbreviations as follows: V+ (virulent), V- (non-virulent), C+ (cytotoxic), C- (non-cytotoxic).



**Fig. 4.** Domain architecture of the F-type MARTX toxin identified in *V. lentus* TO6\_11. Schematic representation of the protein architecture of MARTX toxins. Different architectures found in *V. splendidus* stains associated with oysters (Bruto et al., 2018) are compared to the MARTX<sub>TO6\_11</sub>. Reference bar indicates a length of 500 aa. Strains included in the graph are: *V. splendidus* ZF-41; *V. splendidus* 5S-272; *V. splendidus* 1F-55; *V. splendidus* 5S-101; *V. lentus* TO6\_11.



**Fig. 5.** *Vibrio*-host cell interaction by fluorescent microscopy. Hemocyte monolayers adhered to glass coverslips were incubated with fluorescently labeled *Vibrio* strains at a MOI of 50:1 for 3 h. Actin filaments were stained with fluorescent-phalloidin (red), chromatin with TO-PRO iodide® (blue) and *Vibrio* cells expressing GFP appear in green. Scale bar = 20  $\mu$ m. White arrows indicate examples of rounded hemocytes. The virulence/cytotoxicity profile of each strain is indicated next to the strain name: V+: virulent, V-: non-virulent, C-: cytotoxic, C+: non-cytotoxic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Seasonal dynamics of virulence factor prevalence in oyster-associated *Vibrio* communities. Seasonal detection frequencies (%) of the T6SS (*hcp* gene, blue) and MARTX toxin (*rtxA* gene, red) were determined by PCR across four seasons. Data points represent the observed frequencies for each season, connected by trend lines to illustrate temporal variation. The number of *Vibrio* isolates analyzed is indicated next to each season. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## References

- Abarca, A., Lira, G., Bakit, J., 2025. Pacific oyster (*Crassostrea gigas*) aquaculture production in Chile: a review. *Lat. Am. J. Aquat. Res.* 53, 39–55. <https://doi.org/10.3856/vol53-issue1-fulltext-3271>.
- Ahmed, N., Azab, M., Enany, S., et al., 2024. Draft genome sequence of novel Candidatus Ornithobacterium hominis carrying antimicrobial resistance genes in Egypt. *BMC Microbiol.* 24, 47. <https://doi.org/10.1186/s12866-023-03172-6>.
- Aly, S.M., Fathi, M., 2024. Advancing aquaculture biosecurity: a scientometric analysis and future outlook for disease prevention and environmental sustainability. *Aquac. Int.* 32, 8763–8789. <https://doi.org/10.1007/s10499-024-01589-y>.
- ASAB, 2020. Guidelines for the treatment of animals in behavioural research and teaching. *Anim. Behav.* 159, I–XI. <https://doi.org/10.1016/j.anbehav.2019.11.002>.
- Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., Polz, M.F., Roux, F.L., 2017. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J.* 11, 1043–1052. <https://doi.org/10.1038/ismej.2016.162>.
- Bruto, M., Labreuche, Y., James, A., Piel, D., Chenivesse, S., Petton, B., Polz, M.F., Roux, F.L., 2018. Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations. *ISME J.* 12, 2954–2966. <https://doi.org/10.1038/s41396-018-0245-3>.
- Cianfanelli, F.R., Monlezun, L., Coulthurst, S.J., 2016. Aim, load, fire: the type VI secretion system, a bacterial nanoweapon. *Trends Microbiol.* 24, 51–62. <https://doi.org/10.1016/j.tim.2015.10.005>.
- Coulthurst, S.J., 2013. The type VI secretion system – a widespread and versatile cell targeting system. *Res. Microbiol.* 164, 640–654. <https://doi.org/10.1016/j.resmic.2013.03.017>.
- de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., Haffner, P., Degremont, L., Charrière, G.M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., Leroy, M.A., Pérignon, A., Régle, D., Morga, B., Alunno-Bruscia, M., Boudry, P., Roux, F.L., Destoumieux-Garzon, D., Gueguen, Y., Mitta, G., 2018. Immune-suppression by OshV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat. Commun.* 9, 4215. <https://doi.org/10.1038/s41467-018-06659-3>.
- Dégremont, L., Morga, B., Maurouard, E., Travers, M.-A., 2021. Susceptibility variation to the main pathogens of *Crassostrea gigas* at the larval, spat and juvenile stages using unselected and selected oysters to OshV-1 and/or *V. aestuarianus*. *J. Invertebr. Pathol.* 183, 107601. <https://doi.org/10.1016/j.jip.2021.107601>.
- Destoumieux-Garzon, D., Duperruy, M., Vanhove, A.S., Schmitt, P., Wai, S.N., 2014. Resistance to antimicrobial peptides in vibrios. *Antibiotics* 3, 540–563. <https://doi.org/10.3390/antibiotics3040540>.
- Destoumieux-Garzon, D., Canesi, L., Oyanedel, D., Travers, M., Charrière, G.M., Pruzzo, C., Vezzulli, L., 2020. *Vibrio*–bivalve interactions in health and disease. *Environ. Microbiol.* 22, 4323–4341. <https://doi.org/10.1111/1462-2920.15055>.
- Destoumieux-Garzon, D., Montagnani, C., Dantan, L., de Nicolas, N.S., Travers, M.-A., Duperruy, L., Charrière, G.M., Toulza, E., Mitta, G., Cosseau, C., Escoubas, J.-M., 2024. Cross-talk and mutual shaping between the immune system and the microbiota during an oyster's life. *Philos. Trans. R. Soc. B* 379, 20230065. <https://doi.org/10.1098/rstb.2023.0065>.
- Erken, L., Lutz, C., McDougald, D., 2013. The rise of pathogens: predation as a factor driving the evolution of human pathogens in the environment. *Microb. Ecol.* 65 (4), 860–868. <https://doi.org/10.1007/s00248-013-0189-0>.
- Eshwar, A.K., Guldimann, C., Oevermann, A., Tasara, T., 2017. Cold-shock domain family proteins (Csp) are involved in regulation of virulence, cellular aggregation, and flagella-based motility in *Listeria monocytogenes*. *Front. Cell. Infect. Microbiol.* 7, 453. <https://doi.org/10.3389/fcimb.2017.00453>.
- Fox, M., Christley, R., Lupo, C., Moore, H., Service, M., Campbell, K., 2020. Preventing and mitigating farmed bivalve disease: a Northern Ireland case study. *Aquac. Int.* 28, 2397–2417. <https://doi.org/10.1007/s10499-020-00597-y>.
- Freire, J.M.S., Farias, N.D., Hegaret, H., da Silva, P.M., 2023. Morphological and functional characterization of the oyster *Crassostrea gasar* circulating hemocytes: cell types and phagocytosis activity. *Fish Shellfish Immunol. Rep.* 4, 100089. <https://doi.org/10.1016/j.fsirep.2023.100089>.
- Grootjans, S., Hassannia, B., Delrue, I., Goossens, V., Wiernicki, B., Dondelinger, Y., Bertrand, M.J.M., Krysko, D.V., Vuylsteke, M., Vandenaabeele, P., Berghe, T.V., 2016. A real-time fluorometric method for the simultaneous detection of cell death type and rate. *Nat. Protoc.* 11, 1444–1454. <https://doi.org/10.1038/nprot.2016.085>.
- Jana, B., Salomon, D., 2019. Type VI secretion system: a modular toolkit for bacterial dominance. *Future Microbiol.* 14, 1451–1463. <https://doi.org/10.2217/fmb-2019-0194>.
- Kim, B.S., 2018. The modes of action of MARTX toxin effector domains. *Toxins* 10, 507. <https://doi.org/10.3390/toxins10120507>.

- King, W.L., Siboni, N., Kahlke, T., Green, T.J., Labbate, M., Seymour, J.R., 2019. A new high throughput sequencing assay for characterizing the diversity of natural *Vibrio* communities and its application to a Pacific oyster mortality event. *Front. Microbiol.* 10, 2907. <https://doi.org/10.3389/fmicb.2019.02907>.
- Kolmogorov, M., Yuan, J., Lin, Y., Pevzner, P.A., 2019. Assembly of long, error-prone reads using repeat graphs. *Nature biotechnology* 37 (5), 540–546. <https://doi.org/10.1038/s41587-019-0072-8>.
- Kudryashov, D.S., Cordero, C.L., Reisler, E., Satchell, K.J.F., 2008. Characterization of the enzymatic activity of the actin cross-linking domain from the *Vibrio cholerae* MARTX Vc toxin\*. *J. Biol. Chem.* 283, 445–452. <https://doi.org/10.1074/jbc.m703910200>.
- Lasa, A., di Cesare, A., Tassistro, G., Borello, A., Gualdi, S., Furones, D., Carrasco, N., Cheslett, D., Brechon, A., Paillard, C., Bidault, A., Pernet, F., Canesi, L., Edomi, P., Pallavicini, A., Pruzzo, C., Vezzulli, L., 2019 Dec. Dynamics of the Pacific oyster pathobiota during mortality episodes in Europe assessed by 16S rRNA gene profiling and a new target enrichment next-generation sequencing strategy. *Environ. Microbiol.* 21 (12), 4548–4562. <https://doi.org/10.1111/1462-2920.14750>.
- Li, Y.F., Chen, Y.W., Xu, J.K., et al., 2019. Temperature elevation and *Vibrio cyclitrophicus* infection reduce the diversity of haemolymph microbiome of the mussel *Mytilus coruscus*. *Sci. Rep.* 9, 16391. <https://doi.org/10.1038/s41598-019-52752-y>.
- Limthammahisorn, S., Brady, Y.J., Arias, C.R., 2008 Jan. Gene expression of cold shock and other stress-related genes in *Vibrio vulnificus* grown in pure culture under shellstock temperature control conditions. *J. Food Prot.* 71 (1), 157–164. <https://doi.org/10.4315/0362-028x-71.1.157>
- Lokmer, A., Mathias Wegner, K., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J.* 9 (3), 670–682. <https://doi.org/10.1038/ismej.2014.160>.
- Mannion, A., Shen, Z., Buckley-Jordan, E., Garcia, A., Lyons, C., Lee, Y., Boucher, M., Carrasco, S., Gilmore, M.S., Fox, J.G., 2025. Draft genome sequences of *Corynebacterium mastitidis* strains isolated from ocular surface of CD36-knockout mice (B6.129S1-Cd36tm1Mfe/J) with keratitis. *Microbiol. Resource Announcem.* 14 (1), e0056224. <https://doi.org/10.1128/mra.00562-24>.
- Matz, C., Kjelleberg, S., 2005. Off the hook—how bacteria survive protozoan grazing. *Trends Microbiol.* 13 (7), 302–307. <https://doi.org/10.1016/j.tim.2005.05.009>.
- Meuskens, I., Saragliadis, A., Leo, J.C., Linke, D., 2019. Type V secretion systems: an overview of passenger domain functions. *Front. Microbiol.* 10, 1163. <https://doi.org/10.3389/fmicb.2019.01163>.
- Oyanedel, D., Labreuche, Y., Bruto, M., Amraoui, H., Robino, E., Haffner, P., Rubio, T., Charriere, G.M., Roux, F.L., Destoumieux-Garzon, D., 2020. *Vibrio splendidus* O- antigen structure: a trade-off between virulence to oysters and resistance to grazers. *Environ. Microbiol.* 22, 4264–4278. <https://doi.org/10.1111/1462-2920.14996>.
- Oyanedel, D., Rojas, R., Brokordt, K., Schmitt, P., 2022. *Crassostrea gigas* oysters from a non-intensive farming area naturally harbor potentially pathogenic *Vibrio* strains. *J. Invertebr. Pathol.* 196, 107856. <https://doi.org/10.1016/j.jip.2022.107856>.
- Oyanedel, D., Lagorce, A., Bruto, M., Haffner, P., Morot, A., Labreuche, Y., Dorant, Y., de Divonne, S.L.F., Delavat, F., Inguibert, N., Montagnani, C., Morga, B., Toulza, E., Chaparro, C., Escoubas, J.-M., Gueguen, Y., Vidal-Dupiol, J., de Lorgeril, J., Petton, B., Degremont, L., Tourbiez, D., Pimpare, L.-L., Leroy, M., Romatif, O., Pouzadoux, J., Mitta, G., Roux, F.L., Charriere, G.M., Travers, M.-A., Destoumieux-Garzon, D., 2023. Cooperation and cheating orchestrate *Vibrio* assemblages and ´ polymicrobial synergy in oysters infected with OsHV-1 virus. *Proc. Natl. Acad. Sci.* 120. <https://doi.org/10.1073/pnas.2305195120>.
- Parks, DH, Imelfort, M, Skennerton, CT, Hugenholtz, P, Tyson, GW, 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 25 (7), 1043–1055. <https://doi.org/10.1101/gr.186072.114>.
- Piel, D., Bruto, M., James, A., Labreuche, Y., Lambert, C., Janicot, A., Chenivresse, S., Petton, B., Wegner, K.M., Stoudmann, C., Blokesch, M., Roux, F.L., 2020. Selection of *Vibrio crassostreae* relies on a plasmid expressing a type 6 secretion system cytotoxic for host immune cells. *Environ. Microbiol.* 22, 4198–4211. <https://doi.org/10.1111/1462-2920.14776>.
- Roux, F.L., Blokesch, M., 2018. Eco-evolutionary dynamics linked to horizontal gene transfer in *Vibrios*. *Ann. Rev. Microbiol.* 72, 1–22. <https://doi.org/10.1146/annurev-micro-090817-062148>.
- Roux, F.L., Davis, B.M., Waldor, M.K., 2011. Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res.* 39, 1004–1013. <https://doi.org/10.1093/nar/gkq852>.
- Rubio, T., Oyanedel, D., Labreuche, Y., Toulza, E., Luo, X., Bruto, M., Chaparro, C., Torres, M., de Lorgeril, J., Haffner, P., Vidal-Dupiol, J., Lagorce, A., Petton, B., Mitta, G., Jacq, A., Roux, F.L., Charriere, G.M., Destoumieux-Garzon, D., 2019. ´ Species-specific mechanisms of cytotoxicity toward immune cells determine the successful outcome of *Vibrio* infections. *Proc. Natl. Acad. Sci.* 116, 14238–14247. <https://doi.org/10.1073/pnas.1905747116>.
- Satchell, K.J.F., 2015. Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins of *Vibrios*. *Microbiol. Spectr.* 3. <https://doi.org/10.1128/microbiolspec.ve-0002-2014>.
- Scanes, E., Siboni, N., Potts, J., Rao, S., Labbate, M., Seymour, J.R., 2025. Temporal and spatial co-occurrence of pacific oyster mortality and increased planktonic *Vibrio* abundance. *IScience* 28 (2), 111674. <https://doi.org/10.1016/j.isci.2024.111674>

- Schwengers, O., Jelonek, L., Dieckmann, M.A., Beyvers, S., Blom, J., Goesmann, A., 2021. Bakta: rapid and standardized annotation of bacterial genomes via alignment- free sequence identification. *Microbial Genomics* 7 (11), 000685. <https://doi.org/10.1099/mgen.0.000685>.
- Servicio Nacional de Pesca y Acuicultura (SERNAPESCA). 2023. Anuario Estadístico de Pesca y Acuicultura 2023. Valparaíso (Chile): Servicio Nacional de Pesca y Acuicultura. Available from: <https://anuario.sernapesca.dataobservatory.net/>.
- Shneider, M., Buth, S., Ho, B., et al., 2013. PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 500, 350–353. <https://doi.org/10.1038/nature12453>.
- Siboni, N., Balaraju, V., Carney, R., Labbate, M., Seymour, J.R., 2016. Spatiotemporal dynamics of *Vibrio* spp. within the Sydney harbour estuary. *Front. Microbiol.* 7, 460. <https://doi.org/10.3389/fmicb.2016.00460>.
- Song, J., Luo, C., Lim, L., Cheong, K.-L., Farhadi, A., Tan, K., 2024. Protein quality of commercially important edible bivalves. *Crit. Rev. Food Sci. Nutr.* 1–12. <https://doi.org/10.1080/10408398.2024.2315446>.
- Soree, M., Delavat, F., Lambert, C., Lozach, S., Papin, M., Petton, B., Passerini, D., D'égremont, L., Heath, D.H., 2022. Life history of oysters influences *Vibrio parahaemolyticus* accumulation in Pacific oysters (*Crassostrea gigas*). *Environ. Microbiol.* 24, 4401–4410. <https://doi.org/10.1111/1462-2920.15996>.
- Suarez, G., Khajanchi, B.K., Sierra, J.C., Erova, T.E., Sha, J., Chopra, A.K., 2012. Actin cross-linking domain of *Aeromonas hydrophila* repeat in toxin A (RtxA) induces host cell rounding and apoptosis. *Gene* 506, 369–376. <https://doi.org/10.1016/j.gene.2012.07.012>.
- Subasinghe, R., Alday-Sanz, V., Bondad-Reantaso, M.G., Jie, H., Shinn, A.P., Sorgeloos, P., 2023. Biosecurity: reducing the burden of disease. *J. World Aquacult. Soc.* 54, 397–426. <https://doi.org/10.1111/jwas.12966>.
- Subsecretaría de Pesca y Acuicultura (SUBPESCA), 2023. *Estado de situación de las principales pesquerías chilenas*. ano. ~ [https://www.subpesca.cl/portal/618/articles-121344\\_recurso\\_1.pdf](https://www.subpesca.cl/portal/618/articles-121344_recurso_1.pdf).
- Takemura, A.F., Chien, D.M., Polz, M.F., 2014. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Front. Microbiol.* 5, 38. <https://doi.org/10.3389/fmicb.2014.00038>.
- van der Olivier, A.S., Jones, L., Vay, L.L., Christie, M., Wilson, J., Malham, S.K., 2020. A global review of the ecosystem services provided by bivalve aquaculture. *Rev. Aquac.* 12, 3–25. <https://doi.org/10.1111/raq.12301>.
- Wan, S.H., Xu, Y., Xu, W., Leung, S.K.K., Yu, E.Y.N., Yung, C.C.M., 2025. Environmental heterogeneity drives ecological differentiation in *Vibrio* populations across subtropical marine habitats. *Environ. Microbiol.* 27 (5). <https://doi.org/10.1111/1462-2920.70107>.
- Wang, X., Liu, J., Liang, J., Sun, H., Zhang, X.-H., 2020. Spatiotemporal dynamics of the total and active *Vibrio* spp. populations throughout the Changjiang estuary in China. *Environ. Microbiol.* 22, 4438–4455. <https://doi.org/10.1111/1462-2920.15152>.
- Wick, R.R., 2018. *Filtlong*. GitHub, San Francisco, CA.
- Wick, RR, Schultz, MB, Zobel, J, Holt, KE, 2015. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* 31 (20), 3350–3352. <https://doi.org/10.1093/bioinformatics/btv383>.
- Wijisman, J.W.M., Troost, K., Fang, J., Roncarati, A., 2019. Global Production of Marine Bivalves. Trends and Challenges. Springer International Publishing, Cham, pp. 7–26. [https://doi.org/10.1007/978-3-319-96776-9\\_2](https://doi.org/10.1007/978-3-319-96776-9_2).
- Woida, P.J., Satchell, K.J.F., 2020. The *Vibrio cholerae* MARTX toxin silences the inflammatory response to cytoskeletal damage before inducing actin cytoskeleton collapse. *Sci. Signal.* 13. <https://doi.org/10.1126/scisignal.aaw9447>.
- Woida, P.J., Kitts, G., Shee, S., Godzik, A., Satchell, K.J.F., 2022. Actin cross-linking effector domain of the *Vibrio vulnificus* F-type MARTX toxin dominates disease progression during intestinal infection. *Infect. Immun.* 90, e00627-21. <https://doi.org/10.1128/iai.00627-21>.
- Zampini, M., Canesi, L., Betti, M., Ciacci, C., Tarsi, R., Gallo, G., Pruzzo, C., 2003. Role for mannose-sensitive hemagglutinin in promoting interactions between *Vibrio cholerae* El Tor and mussel hemolymph. *Appl. Environ. Microbiol.* 69, 5711–5715. <https://doi.org/10.1128/aem.69.9.5711-5715.2003>.
- Zhang, E., Li, Z., Dong, L., Feng, Y., Sun, G., Xu, X., Wang, Z., Cui, C., Wang, W., Yang, J., 2024. Exploration of molecular mechanisms of immunity in the Pacific oyster (*Crassostrea gigas*) in response to *Vibrio alginolyticus* invasion. *Animals* 14, 1707. <https://doi.org/10.3390/ani14111707>.
- Zhang, B., Qiu, Y., Shi, C., Zhang, J., 2025. Development of multiple real-time fluorescent quantitative PCR for *Vibrio* pathogen detection in aquaculture. *Vet. Sci.* 12, 327. <https://doi.org/10.3390/vetsci12040327>