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# Exogenous indole attenuates key virulence traits and pathogenicity of *Vibrio bivalvicida* in scallop larvae and juveniles

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## ABSTRACT

*Vibrio bivalvicida* VPAP30 is a highly virulent pathogen of the scallop *Argopecten purpuratus*, causing severe larval mortalities in hatcheries. Indole, a tryptophan-derived bacterial signaling molecule, has been proposed as a virulence-attenuating agent in vibrios. To propose sustainable alternatives to antibiotics, we evaluated the effect of exogenous indole (100, 200 and 500  $\mu$ M) on multiple virulence traits of *V. bivalvicida* VPAP30, including gene expression, motility, extracellular product (ECP) secretion, proteolytic activity, cytotoxicity toward scallop hemocytes, and pathogenicity in vivo. Transcriptional analyses of virulence-related genes at 4, 8 and 24 h revealed a concentration- and time-dependent modulation, with 500  $\mu$ M indole consistently repressing flagellar and motility-associated genes and suppressing protease secretion after 24 h of exposure. Consistently, 500  $\mu$ M indole impaired swimming motility, reduced ECP protein content by >90 %, and abolished detectable protease activity. Cytotoxicity assays showed that *V. bivalvicida* VPAP30 strain pre-exposed to 500  $\mu$ M indole induced ~50 % less hemocyte lysis compared with untreated controls, and lower indole concentration. Importantly, in vivo infection assays demonstrated that pre-exposure of *V. bivalvicida* VPAP30 to 500  $\mu$ M indole significantly improved survival of both scallop juveniles and larvae, reducing cumulative mortalities to levels comparable with controls. Together, these findings demonstrate that indole attenuates the virulence of *V. bivalvicida* VPAP30 through concentration-dependent suppression of motility and protease secretion, highlighting its potential as a non-antibiotic strategy to control vibriosis and enhance hatchery sustainability in scallop aquaculture.

## KEYWORDS

*Vibrio bivalvicida* - Scallop pathogen - Indole signaling - Virulence modulation - Aquaculture disease control

## 1. Introduction

The scallop *Argopecten purpuratus* is a commercially important bivalve distributed along the coasts of Peru and Chile, from Paita (5.1°S) to Valparaíso (33.1°S) (Mendo et al., 2016). In Chile, the main natural beds were overexploited by the mid-20th century, prompting the development of aquaculture as a viable strategy for sustainable production (Kluger et al., 2019; von Brand et al., 2016). The adoption of suspended cultivation systems and the establishment of hatcheries became critical to secure a consistent supply of spats (Bakit et al., 2023). Despite these advances, scallop larval rearing continues to face critical challenges, particularly during the early developmental stages, where high mortality rates caused by infectious diseases remain a major bottleneck for hatchery productivity (Dubert et al., 2017; Gonzalez-Pazmino et al., 2021; Rojas et al., 2019).

Among the microbial agents responsible for these outbreaks, vibrios represent the most prevalent and impactful pathogens in bivalve hatcheries (Sanches-Fernandes et al., 2022). Several species within this genus have been associated with larval and spat mortalities (Dubert et al., 2017). Among these pathogenic vibrios affecting scallop hatcheries, *Vibrio bivalvicida* VPAP30 has emerged as a highly pathogenic strain. This strain was first isolated during a mass mortality event of *A. purpuratus* larvae (Rojas et al., 2015). Its virulence was confirmed *in vitro*, where both bacterial cells and the thermo-resistant fraction of their extracellular products (ECPs) induced larval mortality (Rojas et al., 2019). A subsequent study has provided insights into the molecular basis of this pathogenicity. Dual host–pathogen transcriptomic analyses revealed that *V. bivalvicida* modulates a suite of genes involved in key virulence-related processes, including chemotaxis and motility, adherence, immune evasion, antiphagocytosis, iron acquisition, secretion systems, and the production of enzymes and toxins (Jeria et al., 2023). These findings suggest that the bacterium undergoes a coordinated transcriptional response to adapt to and exploit the intra-host environment. Nevertheless, further characterization and functional validation of these candidate factors are required to fully elucidate the pathogenic strategies of *Vibrio* species toward bivalve hosts such as scallops.

Virulence in pathogenic bacteria is a tightly regulated process (Midgett and Kull, 2023). Consequently, disrupting key regulatory pathways has been proposed as a promising approach to develop therapeutic strategies that suppress harmful bacterial behaviors without directly affecting bacterial viability, thereby reducing the selective pressure that drives antimicrobial resistance (Darshane Ruwandeeepika et al., 2012; Deekshit et al., 2023; Mishra et al., 2024). In this context, the tryptophan-derived molecule indole serves as an intraspecies, interspecies and interkingdom signaling compound, influencing diverse bacterial processes including biofilm formation, virulence factor production, and antibiotic resistance (Chunxiao et al., 2023). Indole is synthesized by the enzyme tryptophanase (TnaA) in more than 85 bacterial species (Lee and Lee, 2010). While its broad physiological effects are well documented, its precise molecular targets and mechanisms of action remain incompletely understood (Zarkan et al., 2020). In marine environments, indole may also be naturally generated by bacteria through tryptophan catabolism, potentially influencing interactions among marine bacteria and between bacteria and bivalve hosts (Netz and Opatz, 2015).

From a therapeutic perspective, indole has shown potential as a virulence-attenuating agent in *Vibrio* species. In *Vibrio splendidus*, a pathogen of the sea cucumber *Apostichopus japonicus*, endogenous (naturally produced by the bacterium) indole production correlated with reduced expression of virulence-related genes (Zhang et al., 2017). Similarly, pathogenic vibrios from the Harveyi clade exposed to exogenous indole displayed decreased swimming and swarming motility, along with downregulation of the *pirA* and *pirB* toxin genes (Zhang et al., 2022b). Moreover, treatment with indole analogues conferred protection to *Artemia franciscana* larvae against *Vibrio campbellii* infection (Zhang et al., 2022a).

In light of these findings and considering the relevance of virulence-targeted approaches for improving the sustainability of bivalve aquaculture, we investigated the effect of exogenous indole on multiple virulence-related traits of *V. bivalvicida* VPAP30, including the expression of virulence genes, motility, extracellular product production, and cytotoxicity, as well as its impact on pathogenicity *in vivo* through infection trials in scallop juveniles and larvae. Understanding how indole modulates the virulence of *V. bivalvicida* provides new insights into pathogen-host interactions in scallop juveniles and larvae and may help develop innovative, non-antibiotic strategies to enhance aquaculture sustainability of bivalve.

## 2. Materials and methods

### 2.1. Bacterial culture, endogenous indole detection, and growth curve analysis under indole supplementation

*Vibrio bivalvicida* VPAP30 (Rojas et al., 2019) was cultured at 25 °C in Trypticase Soy Agar (TSA) (Difco) supplemented with 0.5 M NaCl (TSAS) and Trypticase Soy Broth (TSB) (Difco) supplemented with 0.5 M NaCl (TSBS). Indole stock solutions (Sigma-Aldrich, EC 204–420–7) were prepared in pure methanol at 100, 200, and 500 mM and added to TSAS at a 1:1000 ratio to obtain final concentrations of 100, 200, and 500 μM.

Endogenous indole production by *V. bivalvicida* VPAP30 was qualitatively assessed using Kovacs reagent (Sigma-Aldrich®). Briefly, *V. bivalvicida* VPAP30 was cultured in TSBS medium without exogenous indole, starting from an initial optical density (OD<sub>600</sub>) of 0.1 and incubated at 25 °C for 24 h. After incubation, 1 mL of culture was centrifuged at 5000 ×g for 5 min at 4 °C, and 500 μL of the resulting supernatant was mixed with an equal volume of Kovacs reagent (1:1, v/v). The mixture was incubated for 15 min in the dark, and 50 μL of the reaction was transferred to a 96-well microplate (Falcon) for spectrophotometric measurement. Indole concentration was quantified at 530 nm (VersaMax) using an indole standard curve ranging from 0 to 250 μM.

For growth curve assays, a single *V. bivalvicida* VPAP30 colony was inoculated into TSBS containing the corresponding indole concentration and incubated at 25 °C for 18 h. Cultures were then diluted in fresh TSBS (with or without indole) to an initial OD<sub>600</sub> of 0.01 in 200 μL and transferred to 96-well plates (Falcon). Plates were incubated at 25 °C for 24 h, and OD<sub>600</sub> was measured hourly to monitor bacterial growth using a VersaMax microplate reader. All wells contained 200 μL of culture, and measurements were taken every hour with continuous orbital shaking between readings to maintain cells in suspension. Blank wells containing only TSBS (with or without indole) were included in each plate and subtracted from all OD<sub>600</sub> values.

### 2.2. Scallop collection and maintenance

All experimental procedures complied with the U.S. National Research Council guidelines for laboratory animal care (“The Guide for the Care and Use of Laboratory Animals,” 2016). For primary cultures of hemocytes, adult *Argopecten purpuratus* scallops (shell length 60–70 mm; *n* = 10) were obtained from the Granja Marina marine store in Recreo, Vina del Mar, Chile. Scallops were acclimated for three days at the Laboratory of Genetics and Molecular Immunology (LGIM), Pontificia Universidad Católica de Valparaíso, in 100 L tanks containing filtered seawater under the following conditions: 10 % daily water exchange, continuous aeration, an 8 h light photoperiod, and stable temperature (~11 °C). For *in vivo* assays, juvenile *Argopecten purpuratus* scallops were obtained from the aquaculture facilities of the Universidad Católica del Norte (UCN) located at the Tongoy Bay, Chile (30°16' S, 71° 35' W). Scallops were transferred to the

Central Marine Culture Laboratory of the UCN (LCCM-UCN) in Coquimbo and acclimatized for one week in 2000 L tanks supplied with filtered, aerated, running seawater (~16 °C), and fed with a diet of 50 % *Isochrysis galbana* and 50 % *Nannochloropsis sp.* ( $6 \times 10^6$  cells/mL/day). Animals were acclimatized for 5 days prior infection assays. For larval bioassays, *A. purpuratus* mature veliger larvae (180–200 µm, 21 days post-fertilization) were obtained from Ostimar hatchery at Tongoy Bay, Coquimbo region, Chile.

### 2.3. Selection of virulence-related genes for *V. bivalvicida* VPAP30

To evaluate the potential modulation of virulence by indole, we first established a panel of virulence-associated genes for *V. bivalvicida* VPAP30 strain. This dataset was constructed using the predicted virulome of *V. bivalvicida* VPAP30, obtained through the VFAnalyzer pipeline applied to its 4546 coding sequences (CDSs) (GenBank GCA\_001188205.1). Given the previously reported toxicity of *V. bivalvicida* VPAP30 extracellular products (ECPs), we next predicted the bacterial secretome by identifying signal peptides in the amino acid sequences of all 4546 CDSs using the PrediSI tool (<http://www.predisi.de/home.html>) (Hiller et al., 2004). Finally, we intersected this dataset with the list of differentially expressed genes (DEGs) identified in *V. bivalvicida* VPAP30 after eight hours of exposure to four families of mature *A. purpuratus* larvae (F8, F9, F10 and F12) (Jeria et al., 2023), thereby, virulence-related genes that are actively modulated during larval infection. We selected 16 virulence-related genes candidates from this dataset to estimate their expression after indole exposure by RT- qPCR (Supplementary Table S1).

### 2.4. RNA extraction, cDNA synthesis and gene expression analysis by qPCR

For gene expression assays, *V. bivalvicida* VPAP30 was cultured in TSAS either without indole or supplemented with 100, 200, or 500 µM indole for 4, 8, and 24 h (in triplicate). After incubation, 1 mL of each culture was centrifuged at 5000 ×g for 15 min at 4 °C. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL TRIzol™ Reagent (Invitrogen) and stored at – 80 °C until RNA extraction. Total RNA was extracted from bacterial lysates in TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions. After isopropanol precipitation and ethanol washing, samples were treated with DNase I for 15 min to remove genomic DNA. RNA was then re-precipitated using 0.3 M sodium acetate (pH 5.2) and isopropanol (1:1 v/v). RNA concentration and purity were determined spectrophotometrically (NanoDrop Technologies), and integrity was verified by 1 % agarose gel electrophoresis in TAE buffer. The cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™), following the manufacturer's protocol, and stored at – 80 °C until further use. Primer pairs targeting 16 candidate virulence factor genes were designed (Supplementary Table S1). Primer efficiency was evaluated by qPCR using six serial dilutions of pooled bacterial cDNA, in triplicate, with the Takyon® qPCR kit (Eurogentec) on a CFX96 Touch™ system (Bio-Rad). The qPCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, and concluded with a dissociation curve from 65 °C to 95 °C at 0.3 °C/s. Primers displaying approximately 100 % efficiency and a single melting peak were selected for downstream analysis. Relative gene expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (Livak and Schmittgen, 2001) and processed with CFX Manager™ Software v3.1.1517.0823. The *gyrA* gene (DNA gyrase subunit A) served as the reference gene. Heatmaps for gene expression analysis were generated using the Clusvis web tool (<http://biit.cs.ut.ee/clustvis/>), with  $\ln(x) + 1$  transformed data and correlation distance applied for hierarchical clustering (Metsalu and Vilo, 2015).

### 2.5. Swimming motility assay

To determine the effect of indole on bacterial motility, we performed a swimming motility assay on soft agar plates (Zhang et al., 2022b). TSAS soft agar was prepared using 0.2 % agar and indole was added to the medium to obtain final concentrations of 0, 100, or 500 µM. An overnight culture of *V. bivalvicida* VPAP30 grown in TSBS was diluted to an OD<sub>600</sub> of 1. Then, five 1 µL spots of the culture were applied sequentially to the center of each indole-containing plate, letting each spot air-dry before the next, with three replicate plates per treatment. Plates were incubated upright (without inversion) at 25 °C for two days. After incubation, swimming halos were photographed using a ChemiDoc™ Imaging System (Bio-Rad), and their diameters were measured with ImageJ v1.53t. The mean halo diameter of the control group without indole was defined as 100 %, and the diameters from the 100 and 500 µM treatments were normalized to this value.

### 2.6. Obtention of extracellular products (ECPs) and assessment of proteolytic activity

Extracellular products (ECPs) were obtained using the cellophane overlay method on solid media (Rojas et al., 2019). Bacteria were first grown in TSBS, either unsupplemented or supplemented with 100, 200, or 500 µM indole, for 3 h at 25 °C with shaking at 100 rpm. In parallel, sterile cellophane discs were prepared by 20 min UV irradiation and placed on TSAS plates containing the corresponding indole concentration. A 200 µL aliquot of bacterial culture was spread on each disc by surface dissemination technique using a Drigalsky spatula, and plates were incubated at 25 °C for 24 h. Bacterial lawns were harvested by washing the cellophane with cold 1× PBS. The suspensions were centrifuged at 5000 ×g for 15 min at 4 °C, and the supernatants were sequentially filtered through 0.45 µm and 0.22 µm membranes and kept at – 80 °C until further use. Total protein in the resulting ECPs was quantified using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The protein profiles of ECPs were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). ECPs obtained from bacterial cultures exposed to 0 (control), 100, 200, and 500 µM indole for 24 h were concentrated by lyophilization, and equal amounts of total protein (20 µg per lane) were mixed with Laemmli sample buffer and heated at 95 °C for 5 min. Samples were resolved on 12 % SDS-PAGE gels under denaturing conditions. Following electrophoresis, gels were stained with SYPRO™ Ruby Protein Gel Stain (Thermo Fisher Scientific) and visualized under UV illumination. Proteolytic activity of ECPs was determined using an azocasein-based enzymatic assay (Hasegawa et al., 2008). Briefly, 20 µg of ECPs were incubated with 0.5 mg of azocasein (A2765, Merck) dissolved in Tris-HCl buffer (pH 8.0) in a final volume of 100 µL. Proteinase K (20 mg/mL, Invitrogen) was included as a positive control. Each condition and time point was assayed in duplicate. Reactions were stopped by adding 150 µL of 10 % (w/v) trichloroacetic acid (TCA) and incubating on ice for 5 min. Samples were centrifuged at 12,000 ×g for 5 min at 4 °C, and 150 µL of the supernatant was mixed with 150 µL of 1 N NaOH in a 96-well plate. Absorbance was measured at 420 nm using a VersaMax microplate spectrophotometer.

## 2.7. Cytotoxicity assays using primary cultures of scallop hemocytes

Hemolymph was withdrawn from the adductor muscle of adult scallops using a 25-gauge syringe kept on ice to prevent hemocyte aggregation. Hemolymph from each individual was used to plate 96-well plates at a density of  $2 \times 10^5$  hemocytes/well, in triplicate. Each scallop served as a biological replicate in every independent assay, covering all experimental conditions. The remaining hemolymph was centrifuged to discard hemocytes, and the resulting plasma was filtered through 0.22  $\mu\text{m}$  membranes. Hemocytes were allowed to adhere to the wells for 1 h at 18 °C. After adhesion, plasma was removed and replaced with 200  $\mu\text{L}$  of SYTOX™ Green (5  $\mu\text{g}/\text{mL}$ ; Molecular Probes) prepared in sterile sea water (ssw). In parallel, overnight cultures of *V. bivalvicida* VPAP30 were grown in TSBS containing 0, 100, or 500  $\mu\text{M}$  indole and subsequently centrifuged to collect the cells. The bacterial pellets were then resuspended in filtered plasma and incubated at 18 °C for 1 h to allow opsonization by plasma factors, thereby enhancing subsequent interactions with hemocytes. *Vibrio* suspensions were then adjusted to an OD600 of 1 ( $\approx 10^9$  colony forming units (CFU/mL) and added to the wells at a multiplicity of infection (MOI) of 50:1. Cytotoxicity was quantified after 3 h by measuring SYTOX Green fluorescence ( $\lambda_{\text{ex}} = 480 \text{ nm}$  /  $\lambda_{\text{em}} = 550 \text{ nm}$ ) using a TECAN microplate reader.

## 2.8. In vivo infection of scallop juveniles and larvae with *V. Bivalvicida* VPAP30

Juvenile scallops (2–3 cm in length) were infected using *Vibrio bivalvicida* VPAP30 that had been pre-cultured overnight on TSAS plates supplemented with 0, 100, or 500  $\mu\text{M}$  indole. Bacterial colonies from each treatment were then inoculated into TSBS containing the same indole concentrations (0, 100, or 500  $\mu\text{M}$ ) and incubated at 20 °C for 6 h to obtain cells in the exponential growth phase. Cultures were subsequently centrifuged at 5000  $\times g$  for 10 min, the supernatant discarded, and the resulting pellets washed and resuspended in ssw. Each suspension was adjusted to a final concentration of  $2.5 \times 10^8$  CFU/mL. For the infection assay, 120 scallops were randomly distributed into four groups (30 individuals per group). Three groups were challenged by injecting 10  $\mu\text{L}$  of *V. bivalvicida* VPAP30 suspension ( $2.5 \times 10^6$  CFU per scallop), pre-cultivated with 0, 100, or 500  $\mu\text{M}$  indole, into the striated adductor muscle using sterile syringes (25G  $\times$  5/8). The fourth group received 10  $\mu\text{L}$  of ssw as a negative control. All scallops were maintained in 6 L aquaria with ssw filtered through 0.22  $\mu\text{m}$  membranes, constant aeration, and daily feeding. Thirty percent of the seawater was renewed daily, and the temperature was maintained at 16 °C. Mortality was recorded daily for seven days post-infection. Each scallop was injected individually with a defined bacterial dose, and therefore each animal constituted an independent biological replicate. Because infection was performed by individual injection rather than by waterborne exposure, no tank-level technical replicates were applicable. Injection-based assays reduce tank-level variability since all scallops receive the exact same inoculum, allowing a single tank per treatment to be used without compromising experimental consistency.

For scallop larvae infection assays, groups of 100 larvae were placed in 6-well plates containing 4 mL of ssw with gentle aeration. *V. bivalvicida* VPAP30 cultures were prepared as described above and adjusted to an approximate concentration of  $2.7 \times 10^5$  CFU/mL. Larvae were exposed for 24 h at 18 °C to bacteria pre-cultured in the absence or presence of indole (0 or 500  $\mu\text{M}$ ). Negative controls consisted of larvae incubated in ssw without bacteria. After exposure, larval survival was assessed by inverted microscopy (NIKON, Eclipse 50i, 200 $\times$ ) using motility and ciliary activity as viability criteria as described before (Jeria et al., 2023). Each condition was tested in triplicate.

## 2.9. Statistical analyses

Data normality was evaluated using the Shapiro–Wilk test, and homogeneity of variances was verified with Levene’s test. Non-parametric datasets from qPCR expression analysis, motility assays, ECP proteolytic activity, cell viability and larval bioassays were analyzed using the Kruskal–Wallis test followed by Dunn’s *post hoc* test for multiple comparisons. Survival data from infected juveniles were analyzed by constructing Kaplan–Meier survival curves and calculating cumulative survival rates. All statistical analyses were conducted with GraphPad Prism v10.0.0 (GraphPad Software, Windows).

## 3. Results

### 3.1. Selection of virulence-related genes in *V. Bivalvicida* VPAP30

We assembled a panel of virulence-related genes for *V. bivalvicida* VPAP30 based on three criteria: (i) previously validated virulence factors in pathogenic bacteria, (ii) proteases, metalloproteases, or toxins predicted to be secreted into the extracellular environment, and (iii) candidate genes differentially expressed eight hours after scallop larval infection (Supplementary Table S1). The initial dataset included 145 virulence factors identified by the VFAnalyzer pipeline and 1253 genes with predicted signal peptides according to PrediSI. Of these, 44 genes overlapped between the two categories. Cross-referencing this list with the differentially expressed genes (DEGs) reported by Jeria et al. (2023) resulted in 340 candidates with  $\log_2$  fold-change values ranging from –11.65 to 8.24.

This analysis led us to select 16 virulence factors potentially contributing to the virulence of *V. bivalvicida* VPAP30 (Supplementary Table S1). They encompassed different categories of virulence factors, including: (i) three hemolysin-coregulated proteins belonging to distinct Type VI Secretion Systems—two encoded in chromosomal loci and one on a putative plasmid predicted in the genome; (ii) the Type III Secretion System CesT chaperone, which was upregulated during larval infection; (iii) a thermolabile hemolysin (SGNH family); (iv) several putatively secreted peptidases, such as aminopeptidase N, peptidase Do, a peptidase S1 domain-containing protein, protease 2, neutral metalloproteinase, and alkaline protease; (v) the VI capsular polysaccharide synthesis-associated protein TviB; and (vi) flagellar proteins including FlgM, FlgE, FlgD, and FlgB.

### 3.2. Modulation of virulence-related genes by exogenous indole

Before evaluating transcriptional responses, we first quantified endogenous indole production and verified that indole supplementation did not alter bacterial growth. Endogenous indole production of *V. bivalvicida* VPAP30 was quantified using Kovacs reagent, which reached 12.16  $\mu\text{M}$  after 24 h of culture, which is substantially lower than the exogenous concentrations applied in the indole exposure assays (Supplementary Fig. S1). In parallel, to assess whether

exogenous indole influenced bacterial fitness, growth curves of *V. bivalvicida* VPAP30 were generated in media without or supplemented with 100, 200, or 500  $\mu\text{M}$  indole. No significant differences in growth were observed under any of the tested conditions (Supplementary Fig. S2).

The effect of exogenous indole on the transcription of virulence-associated genes in *Vibrio bivalvicida* VPAP30 was evaluated by exposing growing bacterial cultures to 100, 200, and 500  $\mu\text{M}$  indole for 4, 8, and 24 h. Transcriptional modulation varied according to both indole concentration and exposure time (Fig. 1).

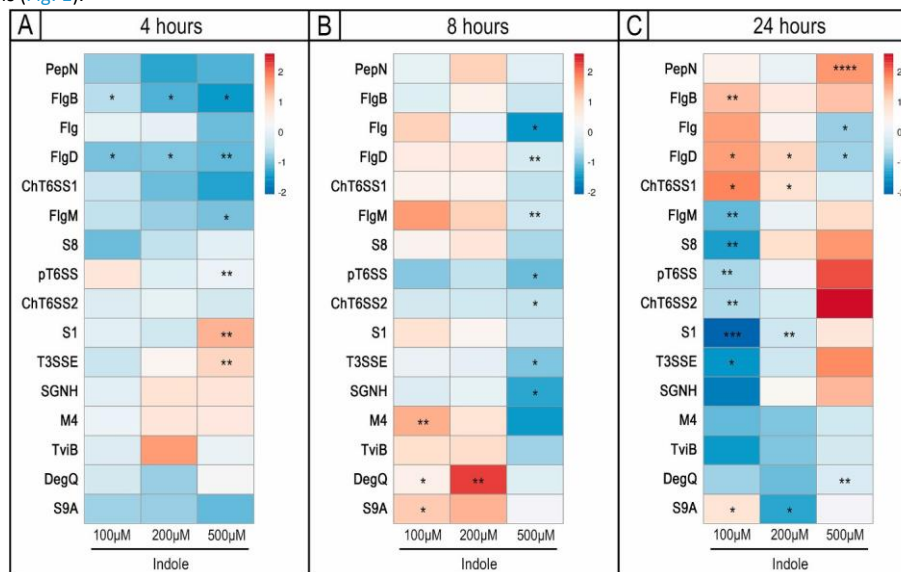
At 4 h of exposure, transcriptional profiles across all concentrations clustered closely together. Indole significantly reduced the expression of flagellar proteins (FlgD and FlgB) at all concentrations, while FlgM was downregulated only at 500  $\mu\text{M}$ . At this highest concentration, a putative Type III secretion system effector (T3SSE) and a protease S1 were upregulated, whereas the the Hcp protein from a plasmid-borne Type VI secretion system (pT6SS) was repressed.

After 8 h of exposure, indole continued to modulate multiple virulence-related proteins (Fig. 1). The S9A family protease, the M4 metalloprotease, and the DegQ protein were significantly upregulated, with the latter also strongly induced at 200  $\mu\text{M}$ . In contrast, 500  $\mu\text{M}$  indole produced a general downregulation, affecting flagellar components (FlgD, FlgB, FlgM), the Hcp protein from both chromosomal (ChT6SS2) and plasmidial (pT6SS) Type VI secretion systems, a putative T3SSE effector, and a SGNH protease. Overall, hierarchical clustering indicated that transcriptional profiles at 4 h and 8 h remained relatively similar across concentrations, although 500  $\mu\text{M}$  consistently triggered more extensive gene modulation (Fig. 1).

By 24 h, transcriptional profiles diverged more clearly with indole concentration, and a distinct clustering pattern emerged (Fig. 1). Profiles from the 100  $\mu\text{M}$  and 200  $\mu\text{M}$  treatments grouped together, whereas the 500  $\mu\text{M}$  condition formed an independent branch. The 100/200  $\mu\text{M}$  cluster displayed induction of Hcp from a chromosomal T6SS not modulated under earlier conditions, along with upregulation of the flagellar protein FlgD; FlgB was upregulated only at 100  $\mu\text{M}$ . Despite clustering together, the 100  $\mu\text{M}$  treatment uniquely repressed several secretion system-, protease-, and flagella-related components (ChT6SS2, pT6SS, SGNH, T3SSE, S1, FlgM) that remained unaltered at 200  $\mu\text{M}$ . Additionally, protease S9A was upregulated at 100  $\mu\text{M}$  but downregulated at 200  $\mu\text{M}$ .

The distinct 500  $\mu\text{M}$  profile at 24 h was characterized by downregulation of flagellar components (FlgD, FlgM) and the protease DegQ, together with upregulation of the proteases PepN and S1 (Fig. 1). This pattern indicates that prolonged exposure to indole (particularly at 100 and 500  $\mu\text{M}$ ) promotes the emergence of strongly contrasted transcriptional signatures in virulence-related genes. The most striking difference was therefore observed between the control (0  $\mu\text{M}$ ) and low/intermediate indole concentrations (100–200  $\mu\text{M}$ ) versus the high indole treatment (500  $\mu\text{M}$ ) (Fig. 1). At 500  $\mu\text{M}$ , genes encoding secretion system components (pT6SS, T3SSE, ChT6SS2) and the SGNH hydrolase were consistently upregulated, along with several proteases (PepN, DegQ, S1, S8, M4).

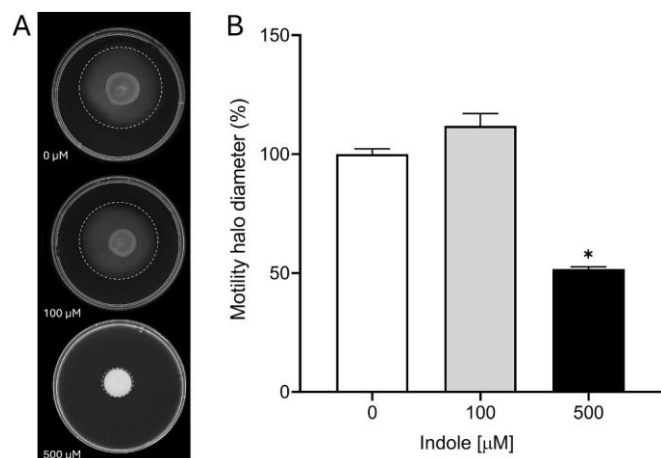
Overall, these results indicate that prolonged exposure (24 h) to high indole levels (500  $\mu\text{M}$ ) promotes a distinct transcriptional program characterized by repression of motility-related genes and activation of proteases and secretion systems, clearly separating the 500  $\mu\text{M}$  treatment from the control and intermediate concentrations (Fig. 1).



**Fig. 1.** Heatmap showing the expression of virulence-related genes in *Vibrio bivalvicida* VPAP30 following exposure to indole. Gene expression profiles are shown for treatments with 100, 200, and 500  $\mu\text{M}$  indole at 4 h (A), 8 h (B), and 24 h (C). Expression levels were normalized to the reference gene *gyrA* and expressed as  $\log_{10}$  fold change relative to the untreated control (0  $\mu\text{M}$  indole) using the  $2^{-\Delta\Delta\text{Ct}}$  method. Asterisks indicate statistically significant differences relative to the control ( $p < 0.05$ ). Data represent mean values from three biological replicates per condition.

### 3.3. Effect of indole on the motility of *Vibrio bivalvicida* VPAP30

Following the observation that transcripts encoding flagellin-related putative virulence factors were modulated by indole, the effect of this compound on the motility of *V. bivalvicida* VPAP30 was evaluated using a motility assay (Fig. 2). The assay revealed that exposure to 100  $\mu\text{M}$  indole did not produce any measurable effect on bacterial motility, whereas 500  $\mu\text{M}$  indole significantly impaired swimming ability (Fig. 2A). After 48 h of incubation, cultures exposed to 500  $\mu\text{M}$  indole exhibited a 48.42 % reduction in the diameter of the swimming halo compared with the untreated control. In contrast, the lowest concentration tested, 100  $\mu\text{M}$ , did not significantly modify the motility pattern of the bacteria (Fig. 2B).



**Fig. 2.** Effect of indole on the motility of *Vibrio bivalvicida* VPAP30. Bacteria were cultured for 48 h on TSBS agar supplemented with or without indole (0.2 % agarose). The 0 μM condition represents *V. bivalvicida* VPAP30 cultured without indole, and 100 μM and 500 μM correspond to VPAP30 grown with those indole concentrations. (A) Representative images of motility halos (outlined with a white dashed line) for 0, 100 μM and 500 μM indole. (B) Quantification of *V. bivalvicida* VPAP30 motility halo diameters ( $n = 3$ ). Error bars represent the standard error of the mean (SEM). Asterisks above bars indicate statistically significant differences compared to the control ( $*p < 0.0001$ ).

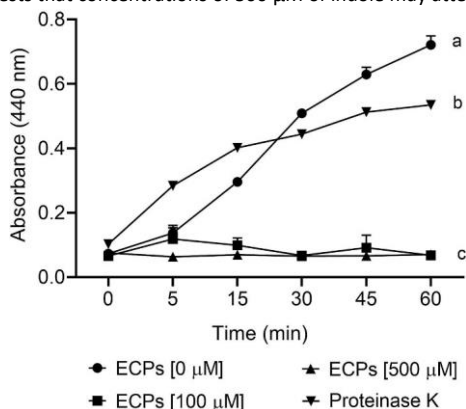
### 3.4. Impact of indole on the extracellular products (ECPs) and protease activity of *V. bivalvicida* VPAP30

Given the transcriptional modulation observed in genes encoding proteases and secretion system components, we next evaluated the impact of exogenous indole on the production of extracellular products (ECPs) by *V. bivalvicida* VPAP30 and their associated protease activity.

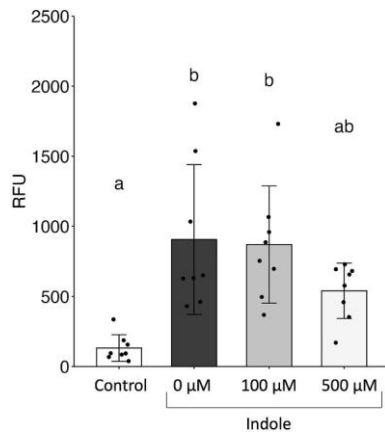
After 24 h of culture, the total protein content of ECPs was  $0.527 \pm 0.021$  mg/mL in media without indole. This protein concentration decreased to  $0.057 \pm 0.006$  mg/mL and  $0.043 \pm 0.006$  mg/mL in media supplemented with 100 and 500 μM indole, respectively. Consistent with this reduction, the electrophoretic profile of ECPs from indole-treated cultures was completely altered compared to the control, with the 100 μM and 500 μM treatments displaying distinct protein band patterns indicative of major changes in overall protein composition induced by indole exposure (Supplementary Fig. S3). To evaluate the potential functional impact of indole exposure to virulence-associated properties of the ECPs, we measured its protease activity. For this assay, ECPs from indole-treated cultures were normalized to the same protein concentration across samples (20 μg). ECPs from untreated *V. bivalvicida* VPAP30 cultures exhibited protease activity comparable to that of proteinase K at 20 mg/mL, whereas no significant activity was detected in ECPs from bacteria cultured with 100 μM or 500 μM indole (Fig. 3).

### 3.5. Effect of indole over the cytotoxicity of *Vibrio bivalvicida* VPAP30 in primary cultures of scallop hemocytes

To evaluate the cytotoxic activity of *V. bivalvicida* VPAP30, primary cultures of scallop hemocytes were incubated with bacteria previously exposed to 0, 100, or 500 μM indole for 24 h. Hemocyte lysis was quantified using Sytox Green after 3 h of contact (Fig. 4). *V. bivalvicida* VPAP30 pre-exposed to 0 or 100 μM indole induced a significant increase in hemocyte lysis compared to the control (Dunn's test,  $p_{\text{adj}} = 0.0026$ ). In contrast, an apparent reduction of nearly 50 % in the Sytox Green signal was observed with bacteria pre-exposed to 500 μM indole (Dunn's test,  $p_{\text{adj}} = 1$ ), compared to *V. bivalvicida* VPAP30 alone. These suggests that concentrations of 500 μM of indole may attenuate the cytotoxic activity of *V. bivalvicida* VPAP30 toward host immune cells.



**Fig. 3.** Effect of indole on protease activity in extracellular products (ECPs) of *Vibrio bivalvicida* VPAP30. ECPs were obtained from *V. bivalvicida* VPAP30 cultures grown with 0, 100, or 500 μM indole, normalized to equal protein concentrations (20 μg), and tested with azocasein. Absorbance at 440 nm was measured over 60 min and compared with proteinase K (positive control). Each condition was tested in triplicate. Data represents SD; different lowercase letters indicate statistically significant differences at 60 min ( $p < 0.05$ ).

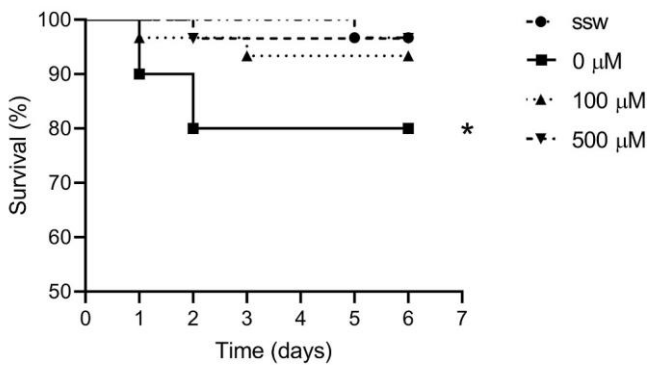


**Fig. 4.** Effect of indole on the cytotoxicity of *Vibrio bivalvicida* VPAP30 toward scallop hemocytes. Control corresponds to hemocytes incubated without bacteria. The 0 μM condition corresponds to *V. bivalvicida* VPAP30 cultured without indole, and the 100 μM and 500 μM conditions to *V. bivalvicida* VPAP30 cultured with indole at those concentrations. Hemocyte lysis was quantified using Sytox Green after 3 h of co-incubation with *V. bivalvicida* VPAP30 pre-exposed to indole for 24 h. Bars represent mean ± SD; different lowercase letters indicate statistically significant differences among treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

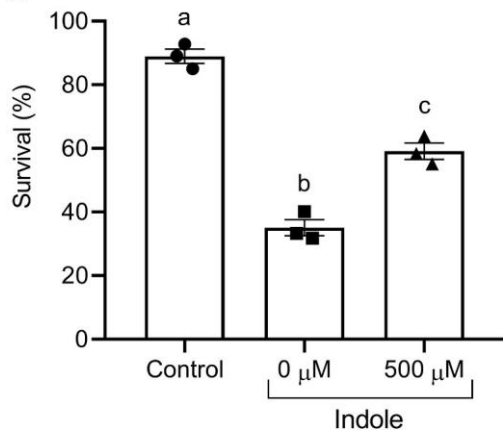
3.6. Effect of indole over the pathogenicity of *Vibrio bivalvicida* VPAP30 in scallop juveniles and larvae

To assess whether the virulence-attenuating effects of indole observed *in vitro* translated into protection *in vivo*, we conducted infection trials in scallop juveniles and larvae (Fig. 5). Kaplan–Meier survival analysis of juveniles revealed significant differences among treatments (Fig. 5A). Scallops injected with *V. bivalvicida* VPAP30 cultured without indole (0 μM indole) exhibited significantly reduced survival, with cumulative mortality reaching ~20 % by day 6 post-infection ( $p < 0.05$  vs. control). In contrast, juveniles challenged with bacteria pre-cultivated in 100 μM or 500 μM indole showed survival rates comparable to the negative control group (ssw-injected scallops), with no significant differences detected among these groups.

A



B



**Fig. 5.** Indole effect on the pathogenicity of *Vibrio bivalvicida* VPAP30 in scallop juveniles and larvae. (A) Kaplan–Meier survival curves of juveniles injected with *V. bivalvicida* VPAP30 suspensions pre-cultured with 0, 100, or 500 μM indole ( $2.5 \times 10^6$  CFU/scallop); control group was injected with sterile seawater (ssw). (B) Survival of larvae challenged for 24 h with *V. bivalvicida* VPAP30 pre-cultured with 0 or 500 μM indole ( $2.7 \times 10^5$  CFU mL<sup>-1</sup>); the control group consisted of larvae incubated only in sterile seawater (ssw, without bacteria). Data are mean ± SEM (n = 3); different letters above bars indicate statistically significant differences ( $p < 0.05$ ).

A similar trend was observed in the larval assays conducted over 24 h (Fig. 5B). Larvae exposed to untreated *V. bivalvicida* (0  $\mu\text{M}$  indole) showed a pronounced decrease in survival, reaching approximately 35 % by the end of the experiment. In contrast, when larvae were challenged with bacteria previously cultured in the presence of 500  $\mu\text{M}$  indole, survival increased significantly to ~60 % ( $p < 0.05$ ). Control larvae that were not exposed to bacteria maintained survival levels close to 90 %, confirming that the observed differences were specifically related to the effect of indole on bacterial virulence. Statistical analysis also confirmed that larval survival in the 0  $\mu\text{M}$  treatment was significantly lower than in the 500  $\mu\text{M}$  treatment, and that both were significantly different from the uninfected control ( $p < 0.05$ ).

#### 4. Discussion

*Vibrio* infections in cultured bivalves represent a persistent challenge, where understanding the determinants of pathogen virulence is essential for developing novel disease management and therapeutic strategies. In this study, we examined how exogenous indole modulates virulence traits in the scallop pathogen *Vibrio bivalvicida* VPAP30, including the expression of virulence-related genes, motility, extracellular product (ECP) secretion, *in vitro* cytotoxicity toward scallop hemocytes and *in vivo* pathogenicity against scallop juveniles and larvae. This framework provides an initial assessment of pathogenicity and has been used successfully to functionally validate the contribution of genetic determinants to virulence in bivalve-pathogenic vibrios (Le Roux et al., 2007; Oyanedel et al., 2020; Piel et al., 2020; Rahmani et al., 2021).

Indole exposure produced concentration- and time-dependent shifts in the expression of multiple virulence factors, with clear transcriptional differences between treatments with 100  $\mu\text{M}$  and 500  $\mu\text{M}$  at 24 h. This transcriptional divergence provided an opportunity to link gene-level changes to phenotypic outputs relevant to pathogenesis. Although the transcriptional differences detected were modest in magnitude, they are consistent with the regulatory patterns previously described for *Vibrio* species exposed to indole, where subtle gene modulation has also been associated with clear phenotypic attenuation (Mueller et al., 2009; Zhang et al., 2017; Zhang et al., 2022a). In our system, these effects likely arise from the combined contribution of the genes analyzed together with additional indole-responsive regulatory pathways not captured within our RT-qPCR panel. Consistent with reports in other vibrios (Zhang et al., 2022a), indole strongly inhibited *V. bivalvicida* VPAP30 motility with a pronounced reduction at 500  $\mu\text{M}$ . This effect coincided with the downregulation of *flgD* and *flaE*, encoding key structural components of the hook assembly and polar flagellin, respectively. Similar indole-mediated reductions in swimming and swarming motility have been observed in *V. tasmaniensis* LGP32 and *V. crassostreae* J2–9, with additional inhibition of biofilm formation in the latter (Zhang et al., 2022b). Comparable responses have been reported across twelve Harveyi-clade strains, where indole treatment decreased biofilm formation, swimming and swarming motility, and downregulated virulence-associated toxin genes in *V. parahaemolyticus* and *V. campbellii* (Zhang et al., 2022b).

Beyond its effect on motility, indole markedly altered the composition and activity of *V. bivalvicida* VPAP30 extracellular products (ECPs), most notably by suppressing protease activity. This is relevant because the heat-stable fraction of *V. bivalvicida* ECPs alone can cause disease in *Argopecten purpuratus* larvae, leading to up to 40 % mortality within 48 h and typical vibriosis lesions such as velum disruption and digestive gland necrosis (Rojas et al., 2019). Comparable protease-dependent pathogenicity has been reported for *V. europaeus*, *V. neptunius*, and *V. splendidus* (Dubert et al., 2017) and for *V. tubiashii*, where metalloprotease inhibition reduces larval toxicity (Hasegawa et al., 2008). In our study, protease-related genes were differentially modulated by indole, showing overall downregulation at 100  $\mu\text{M}$  and upregulation at 500  $\mu\text{M}$ . However, these transcriptional changes alone cannot account for the complete inhibition of protease activity in *V. bivalvicida* ECPs, an effect that warrants further investigation.

We next examined the direct effects of *V. bivalvicida* VPAP30 on hemocytes *in vitro* to assess whether changes in gene expression and ECP activity affected its cytotoxic potential. *V. bivalvicida* VPAP30 caused strong hemocyte lysis compared to the control, an effect unchanged at 100  $\mu\text{M}$  indole, indicating that intermediate concentrations do not alter cytotoxicity and that the modulated virulence factors (ChT6SS2, pT6SS, SGNH, S1, S8, FlgM, S9A, FlgB, FlgD, ChT6SS1) contribute little to this phenotype. In contrast, pre-exposure to 500  $\mu\text{M}$  indole reduced Sytox Green fluorescence by ~50 % relative to untreated bacteria, yielding lysis levels not significantly higher than the control and indicating partial attenuation of cytotoxicity. The persistence of some cytotoxicity at 500  $\mu\text{M}$ , despite reduced motility and protease activity, suggests that (i) motility is not essential in this *in vitro* setting—unlike *in vivo*, where bacterial movement toward host cells is required—and (ii) proteolytic secretion is not necessary for *V. bivalvicida* VPAP30-induced cytotoxicity under these conditions.

*In vivo* assays demonstrated that indole markedly attenuates the virulence of *V. bivalvicida* VPAP30 in both scallop juveniles and *A. purpuratus* larvae. Pre-exposure of bacteria to 100 or 500  $\mu\text{M}$  indole prevented the mortality observed in untreated groups, and larvae challenged with 500  $\mu\text{M}$ -treated bacteria showed significantly higher survival. Although *in vitro* and injection assays capture only part of the infection complexity, they support the *in vivo* evidence obtained here. The immersion assay, which reproduces the natural infection route, further confirms the biological relevance of indole's protective effect, likely linked to reduced bacterial motility and impaired ability to reach swimming larvae. Accordingly, the distinct bacterial concentrations used in juveniles and larvae reflect their contrasting infection routes and stage-specific susceptibility. Because the 100  $\mu\text{M}$  indole treatment did not produce significant changes across multiple phenotypic assays, including motility, extracellular protease activity, hemocyte cytotoxicity, and juvenile survival, the larval infection experiment focused on the 500  $\mu\text{M}$  dose, which consistently showed measurable attenuation. This attenuation aligns with our *in vitro* findings and previous reports showing that indole decreases motility, protease activity, and secretion systems in *V. cholerae* and *V. anguillarum* (Che et al., 2025; Li et al., 2014; Mueller et al., 2009; Saha et al., 2023). Comparable concentration-dependent reductions in virulence have been described for *V. tasmaniensis* LGP32, *V. crassostreae* J2–9, and *V. harveyi*, where indole impaired quorum-sensing-regulated traits and reduced host mortality (Zhang et al., 2022b). Together, these results expand the evidence that indole acts as a conserved bacterial signal that downregulates virulence pathways across *Vibrio* species, reinforcing its potential as a non-antibiotic strategy for disease control in aquaculture. Considering that *V. bivalvicida* VPAP30 was originally isolated from a massive larval mortality outbreak in commercial scallop hatcheries (Rojas et al., 2019), these findings underscore the practical relevance of indole as a preventive agent during this highly vulnerable developmental stage.

In our assays, bacteria were pre-cultured with indole, but the compound was absent during cytotoxicity and infection experiments. Therefore, the observed attenuation likely reflects persistent physiological changes induced by prior exposure rather than a direct inhibitory effect during infection. This indicates that indole can stably reprogram *V. bivalvicida* VPAP30 virulence, supporting its potential as a preventive strategy in scallop hatcheries. Transient treatment of seawater, culture surfaces, or equipment could reduce the virulence of bacteria persisting in biofilms or hard-to-clean areas, consistent with reports that indole-based compounds attenuate biofilm formation and virulence in other Gram-negative pathogens (Golberg et al., 2025). Moreover, indole-producing probiotics could complement standard hygiene practices, improving larval and juvenile survival without antibiotic use, in line with the known bioactive potential of marine indoles as virulence modulators and antibacterial agents (Nett and Opatz, 2015).

Altogether, these results show that indole exerts a multifaceted influence on *V. bivalvicida* VPAP30 virulence, with effects that are trait-specific, concentration-dependent, and sometimes divergent between whole-cell and secreted-product assays. This complexity underscores the need to dissect how

regulatory signaling integrates with virulence gene expression and phenotypic outputs when considering indole or related molecules as modulators of pathogenicity in aquaculture pathogens.

## 5. Conclusion

Our results demonstrate that indole is a potent, dose-dependent modulator of *V. bivalvicida* VPAP30 virulence. At 100  $\mu\text{M}$ , it attenuates hemocyte cytotoxicity without affecting motility, whereas at 500  $\mu\text{M}$  it markedly reduces bacterial motility and suppresses protease activity in the extracellular products. Importantly, *in vivo* assays confirmed that pre-exposure of *V. bivalvicida* VPAP30 to 500  $\mu\text{M}$  indole reduced its pathogenicity in scallop larvae and juveniles, underscoring the biological relevance of the *in vitro* findings. Collectively, these results indicate that indole modulates *V. bivalvicida* VPAP30 virulence through multiple mechanisms, likely involving transcriptional regulation of secretion systems and broad remodeling of the exoproteome. This dual and concentration-dependent response highlights indole as a promising non-antibiotic candidate for virulence attenuation in hatchery settings, although further mechanistic studies, including functional validation and comparative secretome analyses, are needed to fully realize its potential for disease mitigation.

## CRediT authorship contribution statement

**Max Santelices:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Felipe Navarro:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Mauricio Peña-Jaña:** Writing – review & editing, Investigation, Formal analysis. **Roxana Gonzalez:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Rodrigo Rojas:** Investigation, Data curation. **Luz Hurtado:** Methodology, Investigation. **Katherina Brokordt:** Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **Daniel Oyanedel:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Paulina Schmitt:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2025.743561>.

## Data availability

No data was used for the research described in the article.

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