Hemocyte Viability Assay as an Alternative Method for Testing Bacterial Pathogenicity in Bivalves

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ABSTRACT

Bacterial pathogens cause disease outbreaks in hatcheries, leading to larval mortality and significant economic losses. One of the major challenges in studying bacterial pathogenesis in larvae is the limited availability of healthy larvae throughout the year. This seasonal constraint delays testing and impedes progress in understanding bacterial infections, disease management, and the development of effective treatments for bivalve aquaculture. To address this challenge, a hemocyte viability assay was developed as an alternative for testing bacterial pathogenesis. A resazurin-based assay was optimized using eastern oyster hemocytes, and lethal concentration $(LC)_{50}$ values were determined for the bacterial pathogen *Vibrio coralliilyticus* RE22 (121 ± 2 multiplicity of infection, MOI), probiont *Phaeobacter inhibens* S4 (2,157 ± 56 MOI), and several bacterial isolates from shellfish hatcheries. The most virulent isolates had LC_{50} values ranging from 53 to 195 MOI. To validate the assay, the isolates were tested on oyster larvae, confirming that bacterial isolates with LC_{50} <200 MOI against hemocytes also caused larval mortality, while isolates exhibiting LC_{50} values ≥690 MOI showed no adverse effects on oyster larvae. This high-throughput hemocyte viability assay provides a rapid and reliable alternative to larval assays, particularly during the off-season for hatcheries, facilitating year-round research on bacterial pathogenicity in bivalves.

Key words: Bivalve, Larvae, Hemocytes, Resazurin, Oyster, Pathogenicity, Viability

30 **INTRODUCTION**

31 Bivalves are important in fisheries, aquaculture, and ecosystem structure and function
32 (Fernández Robledo et al. 2019; Willer et al. 2021; Mesquita et al. 2023). As filter feeders,
33 bivalves acquire a rich and diverse bacterial commensal microbiota including species of *Vibrio,*
34 *Pseudomonas, Acinetobacter, Photobacterium, Moraxella, Aeromonas, Micrococcus* and *Bacillus*
35 (Kueh and Chan 1985; Zannella et al. 2017). Many of these bacterial species play a beneficial role
36 to the bivalve host, contributing to the digestion of food, including the breakdown of complex
37 carbohydrates and the production of essential amino acids, and the detoxifying of pollutants like
38 heavy metals and organochlorines (Masanja et al. 2023). A few of those bacterial species,
39 however, are pathogenic to bivalves, constituting a leading cause of disease outbreaks in bivalve
40 larviculture systems and resulting in significant mortality and economic losses (Zannella et al.
41 2017). These bacteria can rapidly proliferate in aquaculture environments, often exploiting stress
42 or environmental fluctuations to infect and overwhelm bivalve hosts (Elston et al. 2008; Dubert et
43 al. 2017). Pathogenic *Vibrio* spp. are particularly well-characterized, producing virulence factors
44 like hemolysins, proteases, and ciliostatic toxins causing deciliation, loss of velar epithelium, and
45 abnormal swimming behavior (Tubiash et al. 1965; Dubert et al. 2017; Gómez-Chiarri et al. 2025).

46 Experimental larval challenges are commonly used to identify and characterize bacterial
47 pathogens of bivalve larvae (Estes et al. 2004; Gómez-León et al. 2008; Rojas et al. 2019; Wang
48 et al. 2021). The time-consuming nature of these assays, combined with the limited availability of
49 healthy bivalve larvae throughout the year, presents a major challenge in studying the pathogenesis
50 of bacterial isolates, their effects on the larvae, and the development of effective management

51 strategies for bivalve aquaculture. The seasonality of larval production is further complicated by
52 environmental fluctuations and disease pressures that can eradicate or depress larval cohorts
53 (Elston et al. 2008; Dubert et al. 2017; Gradoville et al. 2018), leading to gaps in supply and
54 challenges for experimental replication and validation. Alternative high-throughput methods that
55 are more consistent, accessible, repeatable, and faster are essential. Assays based on measuring the
56 effect of bacterial pathogens on adult bivalve hemocyte viability may provide such an alternative.

57 Bivalves have an open circulatory system in which hemolymph, containing hemocytes,
58 bathes all the organs before returning to the heart via sinuses. These circulating hemocytes are
59 primarily responsible for defending against pathogens through mechanisms such as phagocytosis
60 and encapsulation (Fisher 1986; Canesi et al. 2002; Gerdol et al. 2018). Additionally, antibacterial
61 effectors, opsonins, nonspecific hydrolytic enzymes, and toxic oxygen intermediates found in
62 bivalve hemolymph coordinate the immune response (Song et al. 2010; Gerdol et al. 2018; Chong
63 2022). Hemocytes can recognize, locate, ingest, transport, and digest foreign particles, playing a
64 central role in phagocytosis, encapsulation, inflammation, and wound repair (Fisher 1986; Gerdol
65 et al. 2018). Hemocyte viability is a measure of the proportion of alive cells to evaluate overall
66 hemocyte health. Viability assays can be assessed based on cellular metabolism, enzyme activity,
67 or cell membrane integrity (Kamiloglu et al. 2020). Double fluorescence staining with membrane-
68 permeable nuclear dyes, such as SYBR, and membrane-impermeable dyes, such as propidium
69 iodide (PI), coupled with fluorescence microscopy or flow cytometry, has been commonly used to
70 determine hemocyte viability, but these assays are not quantitative or require complex
71 instrumentation (Allam et al. 2001). Alternatively, the Alamar Blue assay, also known as the

72 resazurin assay, offers an attractive means of assessing hemocyte viability. This assay relies on the
73 reduction of resazurin to resorufin, a fluorescent compound that serves as an indicator of cellular
74 metabolic activity (O'Brien et al. 2000). Resazurin-based assays have been widely used to measure
75 cell viability and cytotoxicity in a range of biological and environmental systems and several cell
76 types, including bacteria, yeast, fungi, protozoa, and cultured mammalian and piscine cells
77 (O'Brien et al. 2000; Rampersad 2012; Petiti et al. 2025).

78 The goal of this work was to develop a high-throughput hemocyte viability assay for
79 assessing bacterial virulence in bivalves without relying on the seasonal availability of bivalve
80 larvae. The assay workflow involves exposure of adult bivalve hemocytes to serial dilutions of
81 bacteria allowing for bacterial-hemocyte interaction, followed by treatment with antibiotics to
82 eliminate bacterial metabolic activity, and, finally, measurement of hemocyte cellular metabolic
83 activity via reduction of resazurin dye. The use of this assay as a screening tool for pathogens of
84 bivalve larvae was validated using larval bacterial challenge assays. This rapid, high-throughput,
85 plate-based resazurin assay will facilitate the identification and characterization of bivalve
86 bacterial pathogens.

87 **MATERIALS AND METHODS**

88 **Bacteria Isolation and Identification**

89 Bacteria were isolated from larvae of the eastern oyster *Crassostrea virginica* and northern
90 quahog *Mercenaria mercenaria* from four hatcheries located along the Atlantic Coast of the United
91 States. The larvae were rinsed and incubated in filtered sterile seawater (FSSW, 3% sea salts,
92 Instant Ocean) overnight at room temperature (20 to 22°C) with aeration before bacterial isolation.

93 After overnight incubation, swimming larvae were filtered through a 40-µm mesh and washed
94 with FSSW. A 100 µL aliquot of the concentrated larvae was combined with 100 µL FSSW and
95 macerated using a sterile grinder or pipette tip until the sample became homogeneous. A 100 µL
96 aliquot of the larvae homogenate was then taken for serial dilution. Then, 20 µL of each serial
97 dilution was plated onto various media, including a marine yeast peptone (YP30; 0.5 % yeast
98 extract, 0.1% peptone, 3% sea salts, 1.5 % agar; pH 7.6) (Karim et al., 2013), marine agar 2216
99 (BD Difco), seawater tryptone agar (SWT, 0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 3%
100 sea salts, 1.5% agar; pH 7.6), and nutrient-free agar plates (3% sea salts, 1.5% agar; pH 7.6), and
101 incubated for 2-5 days at 27°C. Morphologically distinct colonies were selected and re-streaked
102 onto sterile agar plates to ensure the isolation of pure strains.

103 Bacterial DNA was extracted following a modified protocol (Li et al. 2023). Briefly, an
104 overnight culture (500 µL) of each isolate was harvested and resuspended in DNase-free water
105 with 0.1 g of 0.1 mm glass beads (Sigma-Aldrich, USA). The cells were heated at 80°C at 500 rpm
106 for 10 min, followed by RNase treatment (Qiagen, Germany). Bacterial isolates were identified by
107 16S rRNA sequencing using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and
108 1492R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al. 1991). PCR reactions were
109 prepared in a total volume of 25 µL, comprising 5 µL of extracted DNA, 12.5 µL of PCR
110 mastermix (Thermo Scientific), 1 µL each of forward (27F) and reverse (1492R) primers, and 5.5
111 µL of nuclease-free water. Amplification was performed using the following thermal cycling
112 conditions: initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 seconds,
113 annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds; followed by a final
114 extension at 72°C for 5 min. PCR products were purified using a Qiagen PCR purification kit

115 following the manufacturer's protocol and sequenced using a Applied Biosystems 3500xL Genetic
116 Analyzer (Thermo Fisher Scientific, USA) at the RI-INBRE Centralized Research Core Facility
117 (University of Rhode Island, Kingston, RI). Sequence identification was conducted by comparing
118 obtained sequences to the NCBI 16S reference database using BLAST (Camacho et al. 2009).

119 **Overview of the Hemocyte Viability Assay**

120 The workflow for the hemocyte viability assay is illustrated in Fig. 1. The assay relies on
121 incubating hemocytes isolated from adult eastern oysters (Fig. 1A) with dilutions of the bacterial
122 isolates to allow for bacterial – hemocyte interaction (Fig. 1B). After this period of incubation,
123 the hemocyte – bacterial mix is incubated with antibiotics to kill the bacteria (Fig. 1C). Finally, a
124 resazurin-based dye (CellTiter-Blue, Promega) is added to evaluate hemocyte viability (Fig. 1C).

125 **Preparation of Bacterial Dilutions**

126 Bacterial isolates (Table 1) were cultured overnight on YP30 agar plates at 27°C for 18-24
127 hours. After incubation, individual colonies were picked and transferred to liquid YP30 medium,
128 which was incubated at 27°C with shaking (150 rpm) for 18-24 hours. Following the incubation,
129 the bacterial cultures were centrifuged at 16,000 ´ g for 5 min to pellet the cells. The supernatant
130 was discarded, and the bacterial pellet was washed twice with FSSW. The cell pellet was then
131 resuspended in FSSW, and the bacterial concentration of the stock was adjusted to 108 CFU mL-
132 1 based on optical density (OD_{600}). Two-fold bacterial dilutions were prepared in sterile 96-well
133 plates. The final bacterial concentration in each well was verified by spot-plating appropriate
134 dilutions onto YP30 agar plates and determining colony counts (Karim et al. 2013).

135

136 **Preparation of Oyster Hemocytes**

137 Hemocyte isolation was performed on adult eastern oysters collected from Rhode Island
138 farms using aseptic conditions (Fig. 1A). The oyster shells were scrubbed with a sterilized soft-
139 bristle brush to remove surface biofilm, rinsed in freshwater and sprayed with 70% ethanol. A
140 small notch was made on the edge of the shell at the height of the adductor muscle using flame-
141 sterilized pliers. Next, a sterile 1.5" 21G needle attached to a 3 mL syringe was primed with 100
142 µL of ice-cold FSSW, ensuring that air bubbles were removed from the syringe. The primed needle
143 was inserted into the adductor muscle notch, and approximately 1 mL of hemolymph was extracted
144 from each oyster. The needle was rotated gently during extraction to help remove tissue adhering
145 to the needle. To remove tissue debris, hemolymph samples were passed through a 40 µm mesh
146 filter into a 50 mL sterile Falcon tube kept on ice. The pooled hemolymph of 6-12 oysters was
147 then centrifuged at 600 × g for 3 min at 4°C to pellet the hemocytes. The hemocytes were washed
148 twice in FSSW and finally resuspended in FSSW. Hemocyte concentration and viability were
149 evaluated using a hemocytometer, and the concentration was adjusted to 2.0×10^6 cells mL^{-1}. For
150 heat-killed hemocyte preparations (blank control), half of the total hemocyte suspension volume
151 was placed in a water bath at 90°C for 10 min. Live and heat-killed (HK) hemocyte solutions were
152 kept on ice until used in the assay.

153 **Hemocyte Viability Assay Development**

154 The assay was optimized to determine the appropriate hemocyte and antibiotic
155 concentrations, as well as the incubation times for each phase: hemocyte–bacteria interaction,
156 antibiotic treatment, and resazurin metabolism. The final test conditions for the optimized assay

157 were selected based on the most optimal signal to noise ratio and lowest coefficient of variation.
158 The optimized protocol is as follows. Using black clear-bottom 96-well plates (Corning, USA),
159 50 µL of either live or heat-killed hemocytes were added to designated wells of the plate to achieve
160 a final concentration of 1.0×10^6 cells mL^{-1} (Fig.1B). Fifty µL of serial dilutions of the test
161 bacterial suspensions (each in quadruplicate) were added to wells containing hemocytes to achieve
162 a range of multiplicity of infection (MOI). The MOI was calculated by dividing the bacterial
163 concentration (CFU mL^{-1}) by the hemocyte concentration (cells mL^{-1}). The plates were incubated
164 for 1 hour at room temperature to allow bacterial interaction with the hemocytes, then treated with
165 chloramphenicol (200 µg mL^{-1} final concentration) for an additional three hours to prevent further
166 bacterial metabolism. Next, wells were treated with 10 µL of resazurin reagent (Promega, USA)
167 and the plates were incubated for 20 hours at room temperature. Plates were gently shaken for 10
168 seconds and fluorescence was measured (Fluorometer model; BioTek, USA) at an excitation
169 wavelength of 560 nm and an emission wavelength of 590 nm (Fig. 1C).

170 The relative viability of the hemocytes was determined using the following formula:

171 $$\text{Viability (\%)} = \frac{F_{Experimental} - F_{Blank}}{F_{Control} - F_{Background}} \times 100$$

172 Where:

173 $F_{Experimental}$= fluorescence readings of wells containing hemocytes with bacterial treatment.

174 F_{Blank}= fluorescence readings of wells containing heat-killed hemocytes with bacterial treatments
175 (accounts for background fluorescence as well as potential residual metabolic activity from
176 bacteria that was not eliminated by the antibiotic treatment).

177 $F_{Control}$ = fluorescence readings of wells containing hemocytes without bacterial treatment.

178 $F_{Background}$= fluorescence reading of wells containing heat-killed hemocytes without bacterial
179 treatment.
180 Linear regression analysis was performed to plot the relationship between MOI and relative
181 viability. Bacterial concentrations (expressed as MOI) resulting in a 50% reduction in hemocyte
182 viability (LC_{50}) were determined using the regression equation. All calculations were performed
183 using Microsoft Excel**.**

184 **Larval Viability Assays**

185 Larval viability assays were performed using a previously described protocol (Gómez-
186 León et al. 2008) with modifications. Briefly, 2 mL of 6-8-day-old healthy eastern oyster larvae
187 (veligers) were added to 24-well plates at 75-100 larvae mL^{-1}. Then, bacterial isolates were added
188 at a final concentration of 10^5 CFU mL^{-1} and incubated for 16-20 h at room temperature. Larval
189 mortality was determined by visual examination under a microscope (Leica, 400x magnification).
190 Larvae mortality was calculated as follows:

191 $$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of dead larvae + Number of alive larvae}} \times 100$$

192

193 **RESULTS**

194 **Assay Development and Optimization**

195 To establish a reliable and rapid hemocyte viability assay for assessing bacterial virulence,
196 we explored the use of resazurin dye to measure cell survival following treatment with various
197 bacteria. Several parameters were optimized, including hemocyte concentration (ranging from 2.5
198 $\times 10^5$ to 2×10^6), bacteria – hemocyte interaction time (1 to 5h), antibiotic treatment (ampicillin,

199 gentamycin, penicillin, chloramphenicol), and resazurin – hemocyte incubation times (from 1 to
200 24h). Hemocyte concentrations between 1×10^6 and 2×10^6 cells mL^{-1} were determined to yield
201 reliable and reproducible fluorescence measurements (not shown). A concentration of 200 µg mL^{-1}
202 of the broad-spectrum antibiotic chloramphenicol was determined to effectively inhibit the
203 metabolic activity of bacteria without impacting hemocyte viability (Table 2). Fluorescence
204 readings stabilized after 20 hours of incubation of the hemocytes with the resazurin dye, indicating
205 sufficient time for metabolic conversion and accurate viability measurement. Overall, the assay
206 has a 24 h duration, including the bacteria-hemocyte interaction, antibiotic treatment, and
207 incubation with dye (Fig. 1).

208 **Assay Validation Using Known Pathogenic and Probiotic Bacteria**

209 The assay was validated using the larval pathogen *V. coralliilyticus* RE22 and probiont
210 *Phaeobacter inhibens* S4 (Karim et al., 2013). The calculated LC_{50} values, defined as the
211 multiplicity of infection (MOI, ratio of bacteria to hemocytes) required to reduce hemocyte
212 viability by 50%, were determined to be 121 ± 2 for RE22 and 2,157 ± 56 for *P. inhibens* S4 (Fig.
213 2). The assay was repeated three times, with each bacterial concentration tested in quadruplicate,
214 to calculate inter and intra-assay variability. The coefficient of variation for inter-assay accuracy
215 was determined to be 6.3% and 7.7% and for RE22 and S4, respectively.

216 **Application to Field Isolates**

217 The assay was further applied to evaluate the virulence of several bacterial isolates isolated
218 from oyster and clam larvae (Table 1). LC_{50} values ranged from 53 to 25,389 MOI (Table 3),
219 indicating a wide spectrum of virulence. Among the most virulent isolates, *V. toranzoniae* CH7

220 displayed an LC_{50} of 53 MOI, followed by *V. rotiferianus* CH3 (81 MOI), *V. fortis* CH6 (86 MOI),
221 *V. neptunius* DEN11 (129 MOI), *V. chagasii* CH4 (190 MOI), and *C. lytica* CH30 (195 MOI). In
222 contrast, *V. chagasii* CLAM14 and *T. ascidiaceicola* CLAM15 displayed LC_{50} values > 20,000
223 MOI, suggesting these would be non-pathogenic isolates to eastern oysters.

224 **Comparison of the Hemocyte Viability Assay with the Larval Assay**

225 To assess the relationship between hemocyte viability and larval mortality, the bacterial
226 isolates were tested against eastern oyster larvae in challenge assays. Larvae were exposed to
227 bacterial isolates for 16 – 20 h at a standardized bacterial concentration of 10^5 CFU mL^{-1} (Table
228 2). Oyster larvae challenged with the known pathogen *V. coralliilyticus* RE22 had almost 100%
229 mortality. Similarly, potential pathogenic isolates *V. fortis* CH6 and *V. neptunius* DEN11 also had
230 100% mortality. In addition, *V. chagasii* CH4, *C. lytica* CH30, *V. rotiferianus* CH3, *V. toranzoniae*
231 CH7, and *V. tubiashii* DEN41 had larval survival below 50%, confirming their pathogenicity in
232 both assays. Conversely, larvae treated with putative commensal or probiotic isolates exhibited
233 survival rates of 96-100%, indicating their safety towards larvae. Bacterial isolates demonstrating
234 LC_{50} values below 200 MOI in the hemocyte assay also exhibited significant pathogenicity towards
235 oyster larvae. The correlation between mortality and LC_{50} of the pathogenic isolates was
236 significant, with a Pearson correlation coefficient of 0.84 ($P = 0.0021$) (Fig. 3). The range of MOI
237 for isolates that did not cause mortality (survival >90%) in the 20 hour larval assay, however, was
238 highly variable, ranging from 690 to 23,389, suggesting that the hemocyte assay is more sensitive
239 than the short term larval assay.
240
241

242 **DISCUSSION**

243 This study presents a rapid, high-throughput, and reliable hemocyte viability assay as an
244 alternative to traditional larval assays for assessing bacterial virulence against bivalves. Unlike
245 larval-based methods, which are limited by the seasonal availability of healthy larvae and are time-
246 consuming and variable, this assay provides a consistent and quantifiable measure of bacterial
247 effects on pools of adult hemocytes, which can be accessed year-round. Hence, this assay is an
248 attractive tool to complement traditional larval challenge models for the screening of potential
249 bacterial pathogens and the development of disease management strategies, such as probiotic
250 selection.

251 To our knowledge, this is the first report of using resazurin-based viability measurement
252 in bivalve hemocytes to screen for potential pathogens and probiotics of larval bivalves and
253 quantify bacterial virulence. By using resazurin reduction as a measure for hemocyte viability, the
254 assay enables sensitive quantification of bacterial virulence in a controlled, reproducible format.
255 One initial challenge encountered during the assay development was interference by bacterial
256 reduction of resazurin. This issue was resolved by the addition of chloramphenicol, a broad-
257 spectrum antibiotic, after allowing for bacterial-hemocyte interaction and bacterial pathogenicity,
258 and the inclusion of the killed hemocyte and bacterial control, which helps identify those bacterial
259 isolates for which the antibiotic treatment failed to eliminate bacterial metabolism. Hemocytes
260 were unaffected by the antibiotic treatment step, similar to previous reports (Lacaze et al. 2015;
261 Matozzo et al. 2016). Hence, the optimized assay is capable of measuring hemocyte reduction of
262 resazurin without interference from the bacteria isolates being evaluated.

263 **The Hemocyte Viability Assay was Significantly Correlated with the Larval Viability Assay**

264 To validate the hemocyte viability assay, a collection of 19 bacterial isolates from bivalve
265 larvae was tested using both the hemocyte viability assay and the larval viability assay. The two
266 assays showed a significant correlation for those isolates causing mortality in oyster larvae.
267 Isolates *V. toranzoniae* CH7, *V. rotiferianus* CH3, *V. fortis* CH6, *V. neptunius* DEN11, *V. chagasii*
268 CH4, and *C. lytica* CH30, which showed LC_{50} values of <200 MOI, caused ≥ 30% mortality in
269 oyster larvae, confirming the suitability of the hemocyte viability assay to identify larval
270 pathogens. The taxonomic identification of pathogenic isolates using the hemocyte viability assays
271 is consistent with previous reports of pathogenicity toward bivalves (Dubert et al. 2017; Gómez-
272 Chiarri et al. 2025). For example, *V. toranzoniae* and several other *V{Citation}ibrio* spp. (*V. lentus*,
273 *V. crassostreae*, and *V. cyclitrophicus*) caused significantly higher hemocyte cytotoxic rates in the
274 scallop, *Argopecten purpuratus* (González et al. 2025 Apr 1). Similarly, *V. rotiferianus* shows
275 pathogenicity in infection models (Harrison et al. 2022), and *V. fortis* has been implicated in the
276 heat stress-induced mortality of *C. gigas* (Green et al. 2019). Additionally, *V. neptunius* has been
277 identified as an opportunistic pathogen that causes disease in oysters and clams (Galvis et al. 2021),
278 while *V. chagasii* was reported as the causative agent of massive larval mortalities in farmed
279 scallops (*A. purpuratus*) in Chile (Urtubia et al. 2023). Lastly, *C. lytica* has been isolated in the
280 eroded mantle epithelium of juvenile paua (*Haliotis iris*) in New Zealand (Diggles and Oliver
281 2005).

282 Similarly, most isolates with LC_{50} values ≥ 690 MOI in the hemocyte viability assay caused
283 no mortality in the 16–20 hour larval assay, indicating overall good concordance between the two
284 assays. Only three of the 19 tested isolates causing mortality in larvae (*V. tubiashii* isolates DEN12

285 and DEN41 and *V. alginolyticus* DEN30) showed levels of hemocyte viability comparable to
286 isolates that did not cause larval mortality (LC_{50} of 4,160, 848, and 12,204 MOI respectively).
287 This suggests that the pathogenicity of these three isolates to larvae may involve virulence
288 mechanisms not fully captured by hemocyte viability alone, such as tissue-specific interactions or
289 factors expressed only *in vivo*, and/or that adult oyster hemocytes have increased capability for
290 eliminating these strains due to immune system maturation and/or priming (Leonessi et al. 2025).
291 Differences in the LC_{50} in the hemocyte viability assay between the two *V. tubiashii* strains, as
292 well as differences between the two *V. chagasii* strains, is consistent with the well-established
293 principle that *Vibrio* pathogenicity is determined at the strain level, with most species exhibiting a
294 diversity of nonpathogenic and pathogenic strains (Estes et al. 2004; Ushijima et al. 2018;
295 Destoumieux-Garzón et al. 2020; Gómez-Chiarri et al. 2025).

296 The wide range of values in the hemocyte viability assay for isolates causing no mortality
297 in larvae (LC_{50} from 690 to 25,395 MOI) also suggests that the hemocyte viability assay may be
298 more sensitive than a larval assay of a similar duration (around 24 hours), and that some of the
299 isolates may require longer incubation periods with the larvae to show differential impacts on
300 survival. A higher sensitivity of hemocytes to bacterial pathogenicity compared to larvae may also
301 be due to the additional immune defenses and barriers present in larvae (e.g., mucosal barriers
302 (Fernández Robledo et al. 2019)) and/or differential interactions of these with the more complex
303 larval microbiome (Yeh et al. 2020; Arfken et al. 2021; Masanja et al. 2023; Destoumieux-Garzón
304 et al. 2024; Takyi et al. 2024). Notably, six of the eight bacterial strains causing no mortality in
305 oysters were isolated from clams. Because our hemocyte assays were performed using oyster
306 hemocytes, it is possible that these isolates exhibit host specificity and are pathogenic to clams.

307 Such host-specific interactions are well documented in Vibrio – bivalve systems, where virulence
308 can vary widely depending on the host species, immune cell type, and ecological context
309 (Destoumieux-Garzón et al. 2020; Gómez-Chiarri et al. 2025).
310

311 **CONCLUSIONS**

312 This study aimed to develop an alternative assay for assessing bacterial
313 pathogenicity in bivalves, utilizing hemocytes isolated from adult oysters as a substitute for oyster
314 larvae. The results from the hemocyte viability assay developed here correlated well with those
315 from traditional larval challenges, offering a rapid, consistent, and effective tool for testing the
316 pathogenicity of bacterial isolates against bivalves. Some potential limitations of this assay include
317 the fact that this *in vitro* assay may not fully capture the complexity of whole-organism responses
318 in larvae, such as tissue-specific effects or systemic immune responses, a limitation common to
319 most high-throughput *in vitro* screening assays (Ghallab 2013). Additionally, some bacterial
320 species or target host cells may exhibit variable sensitivity to chloramphenicol and/or resazurin
321 (Breijyeh et al. 2020; Vieira-da-Silva and Castanho 2023), potentially affecting assay accuracy.
322 Alternate antibiotics, or even antibiotic cocktails, may be needed in such cases. Despite these
323 limitations, the hemocyte viability assay provides a high-throughput tool for pre-screening isolates
324 for potential pathogenicity and helps solve the bottleneck of larval shortages encountered during
325 the off-season, thereby enabling year-round investigation of potential pathogens and the
326 development of disease mitigation tools for hatcheries. Importantly, this assay can be further
327 adapted to assess the pathogenicity of bacterial isolates in hemocytes from other bivalve species,

328 such as clams, scallops, and mussels. This flexibility makes it a valuable tool for investigating
329 host-specific pathogens across a broader range of bivalve species.

330 **Conflict of Interests**

331 The authors declare that they have no competing interests

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343 **REFERENCES**

344 Allam B, Ashton-Alcox KA, Ford SE. 2001. Haemocyte parameters associated with resistance to
345 brown ring disease in *Ruditapes* spp. clams. Dev Comp Immunol. 25(5):365–375.
346 https://doi.org/10.1016/S0145-305X(00)00072-0

347 Arfken A, Song B, Allen SK, Carnegie RB. 2021. Comparing larval microbiomes of the eastern
348 oyster (*Crassostrea virginica*) raised in different hatcheries. Aquaculture. 531:735955.
349 https://doi.org/10.1016/j.aquaculture.2020.735955

350 Breijyeh Z, Jubeh B, Karaman R. 2020. Resistance of Gram-Negative Bacteria to Current
351 Antibacterial Agents and Approaches to Resolve It. Molecules. 25(6):1340.
352 https://doi.org/10.3390/molecules25061340

353 Camacho C et al. 2009. BLAST+: architecture and applications. BMC Bioinformatics. 10(1):421.
354 https://doi.org/10.1186/1471-2105-10-421

355 Canesi L, Gallo G, Gavioli M, Pruzzo C. 2002. Bacteria–hemocyte interactions and phagocytosis
356 in marine bivalves. Microsc Res Tech. 57(6):469–476. https://doi.org/10.1002/jemt.10100

357 Chong RS-M. 2022. Chapter 57 - Molluscan immunology. In: Kibenge FSB, Baldisserotto B,
358 Chong RS-M, editors. Aquaculture Pathophysiology. Academic Press; p 383–392
359 [accessed 2025 Sept 29].
360 https://www.sciencedirect.com/science/article/pii/B9780323954341000577.
361 https://doi.org/10.1016/B978-0-323-95434-1.00057-7

362 Destoumieux-Garzón D et al. 2020. Vibrio–bivalve interactions in health and disease. Environ
363 Microbiol. 22(10):4323–4341. https://doi.org/10.1111/1462-2920.15055

364 Destoumieux-Garzón D et al. 2024. Cross-talk and mutual shaping between the immune system
365 and the microbiota during an oyster's life. Philos Trans R Soc B Biol Sci.
366 379(1901):20230065. https://doi.org/10.1098/rstb.2023.0065

367 Diggles B, Oliver M. 2005. Diseases of Cultured Paua (*Haliotis iris*) in New Zealand. In: Walker
368 P, Lester R, Bondad-Reantaso MG, editors. Diseases in Asian Aquaculture V. Asian
369 Fisheries Society; p 275–287 (Fish Health Section). [accessed 2025 Apr 3].
370 https://www.semanticscholar.org/paper/Diseases-of-Cultured-Paua-(-Haliotis-iris-)-in-
371 New-Diggles/603882d9afdc76701bc5f3de406d34b382386e1c

372 Dubert J, Barja JL, Romalde JL. 2017. New Insights into Pathogenic Vibrios Affecting Bivalves
373 in Hatcheries: Present and Future Prospects. Front Microbiol. 8 [accessed 2025 Apr 11].
374 https://www.frontiersin.orghttps://www.frontiersin.org/journals/microbiology/articles/10.
375 3389/fmicb.2017.00762/full. https://doi.org/10.3389/fmicb.2017.00762

376 Elston RA et al. 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity,
377 environmental drivers, geographic extent and management. Dis Aquat Organ. 82:119–134.
378 https://doi.org/10.3354/dao01982

379 Estes RM, Friedman CS, Elston RA, Herwig RP. 2004. Pathogenicity testing of shellfish hatchery
380 bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. Dis Aquat Organ. 58(2–
381 3):223–230. https://doi.org/10.3354/dao058223

382 Fernández Robledo JA et al. 2019. From the raw bar to the bench: Bivalves as models for human
383 health. Dev Comp Immunol. 92:260–282. https://doi.org/10.1016/j.dci.2018.11.020

384 Fisher WS. 1986. Structure and Functions of Oyster Hemocytes. Brehélin M, editor. Springer.

385 Galvis F et al. 2021. *Vibrio neptunius* Produces Piscibactin and Amphibactin and Both
386 Siderophores Contribute Significantly to Virulence for Clams. Front Cell Infect Microbiol.
387 11 [accessed 2025 Apr 3].
388 https://www.frontiersin.orghttps://www.frontiersin.org/journals/cellular-and-infection-
389 microbiology/articles/10.3389/fcimb.2021.750567/full.
390 https://doi.org/10.3389/fcimb.2021.750567

391 Gerdol M et al. 2018. Immunity in Molluscs: Recognition and Effector Mechanisms, with a Focus
392 on Bivalvia. In: Cooper EL, editor. Advances in Comparative Immunology. Springer
393 International Publishing; p 225–341 [accessed 2022 Sept 30]. https://doi.org/10.1007/978-
394 3-319-76768-0_11. https://doi.org/10.1007/978-3-319-76768-0_11

395 Ghallab A. 2013. In vitro test systems and their limitations. EXCLI J. 12:1024–1026

396 Gómez-Chiarri M et al. 2025. 11 - Vibriosis of bivalves. In: Smolowitz R, editor. Diseases of
397 Bivalves. Academic Press; p 143–162 [accessed 2025 Apr 5].
398 https://www.sciencedirect.com/science/article/pii/B978012820339200005X.
399 https://doi.org/10.1016/B978-0-12-820339-2.00005-X

400 Gómez-León J et al. 2008. Survival of eastern oysters *Crassostrea virginica* from three lines
401 following experimental challenge with bacterial pathogens. Dis Aquat Organ. 79(2):95–
402 105. https://doi.org/10.3354/dao01902

403 González R et al. 2025. Pathogenic impact of *Vibrio* spp. in scallop hemolymph: Implications for
404 health in aquaculture. Aquaculture. 742507.
405 https://doi.org/10.1016/j.aquaculture.2025.742507

406 Gradoville MR, Crump BC, Häse CC, White AE. 2018. Environmental Controls of Oyster-
407 Pathogenic *Vibrio* spp. in Oregon Estuaries and a Shellfish Hatchery. Appl Environ
408 Microbiol. 84(9):e02156-17. https://doi.org/10.1128/AEM.02156-17

409 Green TJ et al. 2019. Simulated Marine Heat Wave Alters Abundance and Structure of Vibrio
410 Populations Associated with the Pacific Oyster Resulting in a Mass Mortality Event.
411 Microb Ecol. 77(3):736–747. https://doi.org/10.1007/s00248-018-1242-9

412 Harrison J et al. 2022. The increased prevalence of *Vibrio* species and the first reporting of *Vibrio*
413 *jasicida* and *Vibrio rotiferianus* at UK shellfish sites. Water Res. 211:117942.
414 https://doi.org/10.1016/j.watres.2021.117942

415 Kamiloglu S, Sari G, Ozdal T, Capanoglu E. 2020. Guidelines for cell viability assays. Food Front.
416 1(3):332–349. https://doi.org/10.1002/fft2.44

417 Karim M et al. 2013. Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster,
418 *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. J Shellfish Res.
419 32(2):401–408. https://doi.org/10.2983/035.032.0220

420 Kueh CS, Chan KY. 1985. Bacteria in bivalve shellfish with special reference to the oyster. J Appl
421 Bacteriol. 59(1):41–47. https://doi.org/10.1111/j.1365-2672.1985.tb01773.x

422 Lacaze E et al. 2015. Genotoxic and immunotoxic potential effects of selected psychotropic drugs
423 and antibiotics on blue mussel (*Mytilus edulis*) hemocytes. Environ Pollut. 202:177–186.
424 https://doi.org/10.1016/j.envpol.2015.03.025

425 Leonessi M et al. 2025. Interactions between the oyster larvae pathogen *Vibrio ostreicida* and the
426 bivalve hosts *Mytilus galloprovincialis* and *Magallana gigas*. Front Immunol. 16 [accessed
427 2025 Dec 1].
428 https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2025.1711477/
429 full. https://doi.org/10.3389/fimmu.2025.1711477

430 Li S et al. 2023. Combination of direct boiling and glass beads increases the purity and accuracy
431 of bacterial DNA extraction. Biotechnol J. 18(11):2300135.
432 https://doi.org/10.1002/biot.202300135

433 Masanja F et al. 2023. Bivalves and microbes: a mini-review of their relationship and potential
434 implications for human health in a rapidly warming ocean. Front Mar Sci. 10 [accessed
435 2025 Nov 24]. https://www.frontiersin.org/journals/marine-

436 science/articles/10.3389/fmars.2023.1182438/full.
437 https://doi.org/10.3389/fmars.2023.1182438

438 Matozzo V et al. 2016. Does the antibiotic amoxicillin affect haemocyte parameters in non-target
439 aquatic invertebrates? The clam *Ruditapes philippinarum* and the mussel *Mytilus*
440 *galloprovincialis* as model organisms. Mar Environ Res. 119:51–58.
441 https://doi.org/10.1016/j.marenvres.2016.05.017

442 Mesquita AF et al. 2023. Marine Bivalves' Ecological Roles and Humans-Environmental
443 Interactions to Achieve Sustainable Aquatic Ecosystems. In: Marine Ecosystems -
444 Biodiversity, Ecosystem Services and Human Impacts. IntechOpen [accessed 2025 Dec
445 15]. https://www.intechopen.com/chapters/86889.
446 https://doi.org/10.5772/intechopen.111386

447 O'Brien J, Wilson I, Orton T, Pognan F. 2000. Investigation of the Alamar Blue (resazurin)
448 fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem.
449 267(17):5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x

450 Petiti J, Revel L, Divieto C. 2025. Optimizing resazurin-based viability assays for P-MSC/TER308
451 cell line to enhance results reliability. BMC Res Notes. 18(1):228.
452 https://doi.org/10.1186/s13104-025-07298-w

453 Rampersad SN. 2012. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function
454 and Cellular Health in Cell Viability Bioassays. Sensors. 12(9):12347–12360.
455 https://doi.org/10.3390/s120912347

456 Rojas R et al. 2019. Isolation and Pathogenic Characterization of *Vibrio bivalvicida* Associated
457 with a Massive Larval Mortality Event in a Commercial Hatchery of Scallop *Argopecten*
458 *purpuratus* in Chile. Front Microbiol. 10:855. https://doi.org/10.3389/fmicb.2019.00855

459 Song L, Wang L, Qiu L, Zhang H. 2010. Bivalve Immunity. In: Söderhäll K, editor. Invertebrate
460 Immunity. Vol. 708. Springer US; p 44–65 (Advances in Experimental Medicine and
461 Biology). [accessed 2024 Nov 27]. http://link.springer.com/10.1007/978-1-4419-8059-
462 5_3. https://doi.org/10.1007/978-1-4419-8059-5_3

463 Takyi E et al. 2024. Probiotic treatment modulates the bacterial microbiome of larval eastern
464 oysters, *Crassostrea virginica*, in hatcheries. Aquaculture. 583:740624.
465 https://doi.org/10.1016/j.aquaculture.2024.740624

466 Tubiash HS, Chanley PE, Leifson E. 1965. Bacillary necrosis, a disease of larval and juvenile
467 bivalve mollusks. I. Etiology and epizootiology. J Bacteriol. 90(4):1036–1044.
468 https://doi.org/10.1128/jb.90.4.1036-1044.1965

469 Urtubia R et al. 2023. First Report, Characterization and Pathogenicity of *Vibrio chagasii* Isolated
470 from Diseased Reared Larvae of Chilean Scallop, *Argopecten purpuratus* (Lamarck,
471 1819). Pathogens. 12(2):183. https://doi.org/10.3390/pathogens12020183

472 Ushijima B et al. 2018. Factors affecting infection of corals and larval oysters by *Vibrio*
473 *coralliilyticus*. PLOS ONE. 13(6):e0199475.
474 https://doi.org/10.1371/journal.pone.0199475

475 Vieira-da-Silva B, Castanho MARB. 2023. Resazurin Reduction-Based Assays Revisited:
476 Guidelines for Accurate Reporting of Relative Differences on Metabolic Status. Molecules.
477 28(5):2283. https://doi.org/10.3390/molecules28052283

478 Wang D et al. 2021. Pathogenesis of experimental vibriosis in blue mussel (*Mytilus edulis*) larvae
479 based on accurate positioning of GFP-tagged *Vibrio* strains and histopathological and
480 ultrastructural changes of the host. Aquaculture. 535:736347.
481 https://doi.org/10.1016/j.aquaculture.2021.736347

482 Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for
483 phylogenetic study. J Bacteriol. 173(2):697–703. https://doi.org/10.1128/jb.173.2.697-
484 703.1991

485 Willer DF, Nicholls RJ, Aldridge DC. 2021. Opportunities and challenges for upscaled global
486 bivalve seafood production. Nat Food. 2(12):935–943. https://doi.org/10.1038/s43016-
487 021-00423-5

488 Yeh H et al. 2020. From Farm to Fingers: an Exploration of Probiotics for Oysters, from
489 Production to Human Consumption. Probiotics Antimicrob Proteins. 12(2):351–364.
490 https://doi.org/10.1007/s12602-019-09629-3

491 Zannella C et al. 2017. Microbial Diseases of Bivalve Mollusks: Infections, Immunology and
492 Antimicrobial Defense. Mar Drugs. 15(6):182. https://doi.org/10.3390/md15060182

493

494

495 Table 1. List of bacterial isolates used in the study.

ISOLATE TESTED	ISOLATION SOURCE	REFERENCE
Algoriphagus yeomjeoni DEN5	*C. virginica*	This study
Cellulophaga lytica CH30	*C. virginica*	This study
Glutamicibacter soli CLAM16	*M. mercenaria*	This study
Marinomonas gallaica CLAM9	*M. mercenaria*	This study
Phaeobacter inhibens S4	*C. virginica*	Karim et al., 2013
Pseudooceanicola nitratireducens NEH7	*C. virginica*	This study
Tenacibaculum ascidiaceicola CLAM15	*M. mercenaria*	This study
Vibrio alginolyticus DEN30	*C. virginica*	This study
Vibrio chagasii CH4	*C. virginica*	This study
Vibrio chagasii CLAM14	*M. mercenaria*	This study
Vibrio coralliilyticus RE22	*C. gigas*	Estes et al., 2004
Vibrio cyclitrophicus CLAM19	*M. mercenaria*	This study
Vibrio fortis CH6	*C. virginica*	This study
Vibrio neptunius DEN11	*C. virginica*	This study
Vibrio rotiferianus CH3	*C. virginica*	This study
Vibrio tasmaniensis CLAM18	*M. mercenaria*	This study
Vibrio toranzoniae CH7	*C. virginica*	This study
Vibrio tubiashii DEN41	*C. virginica*	This study
Vibrio tubiashii DEN12	*C. virginica*	This study

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497

498 Table 2. Antibiotic concentration to prevent or reduce bacterial growth.

Chloramphenicol (ug mL^{-1})	*V. coralliilyticus* RE22	Hemocyte viability
5	+	+
10	+	+
20	+	+
40	+	+
60	+	+
80	+	+
100	+	+
150	+/-	+
200	-	+
300	-	+
400	-	+
500	-	+

499 + = resazurin dye turned into pinkish

500 - = resazurin dye remained bluish

501

502

503 Table 3. Effect of bacterial isolates on adult oyster hemocyte viability and larval mortality. Effect
504 on hemocyte viability is quantified by the concentration of bacteria, as measured by multiplicity
505 of infection (MOI), required to reduce hemocyte viability by 50% (LC_{50}). Effect on larvae is
506 measured as the percent mortality experienced in a larval culture after a 20 h incubation with a
507 concentration of 10^5 CFU ml

ISOLATE TESTED	LC_{50} (MOI) (Average ± SEM)	Mortality (%) (Average ± SEM)
Vibrio fortis CH6	84	0.0 ± 0.0
Vibrio neptunius DEN11	129	0.0 ± 0.0
**Vibrio coralliilyticus* RE22	121 ± 2	0.9 ± 0.9
Vibrio chagasii CH4	190	18.1 ± 0.1
Cellulophaga lytica CH30	195	23.5 ± 6.5
Vibrio rotiferianus CH3	81	23.9 ± 0.7
Vibrio toranzoniae CH7	53	30.1 ± 0.9
Vibrio tubiashii DEN41	848	45.9 ± 1.7
Vibrio tubiashii DEN12	4,160	46.3 ± 8.1
Vibrio alginolyticus DEN30	12,204	79.7 ± 5.0
Vibrio cyclitrophicus CLAM19	927	93.2 ± 2.2
Marinomonas gallaica CLAM9	704	96.4 ± 1.8
Pseudooceanicola nitratireducens NEH7	690	97.6 ± 0.8
Vibrio chagasii CLAM14	25,389	98.6 ± 1.3
Tenacibaculum ascidiaceicola CLAM15	20,315	99.3 ± 0.7
Glutamicibacter soli CLAM16	1,913	100.0 ± 0.0
**Phaeobacter inhibens* S4	2,157 ± 56	100.0 ± 0.0
Vibrio tasmaniensis CLAM18	877	100.0 ± 0.0
Algoriphagus yeomjeoni DEN5	935	100.0 ± 0.0

508 * Known pathogen and probiotics. SEM: Standard error of the mean.

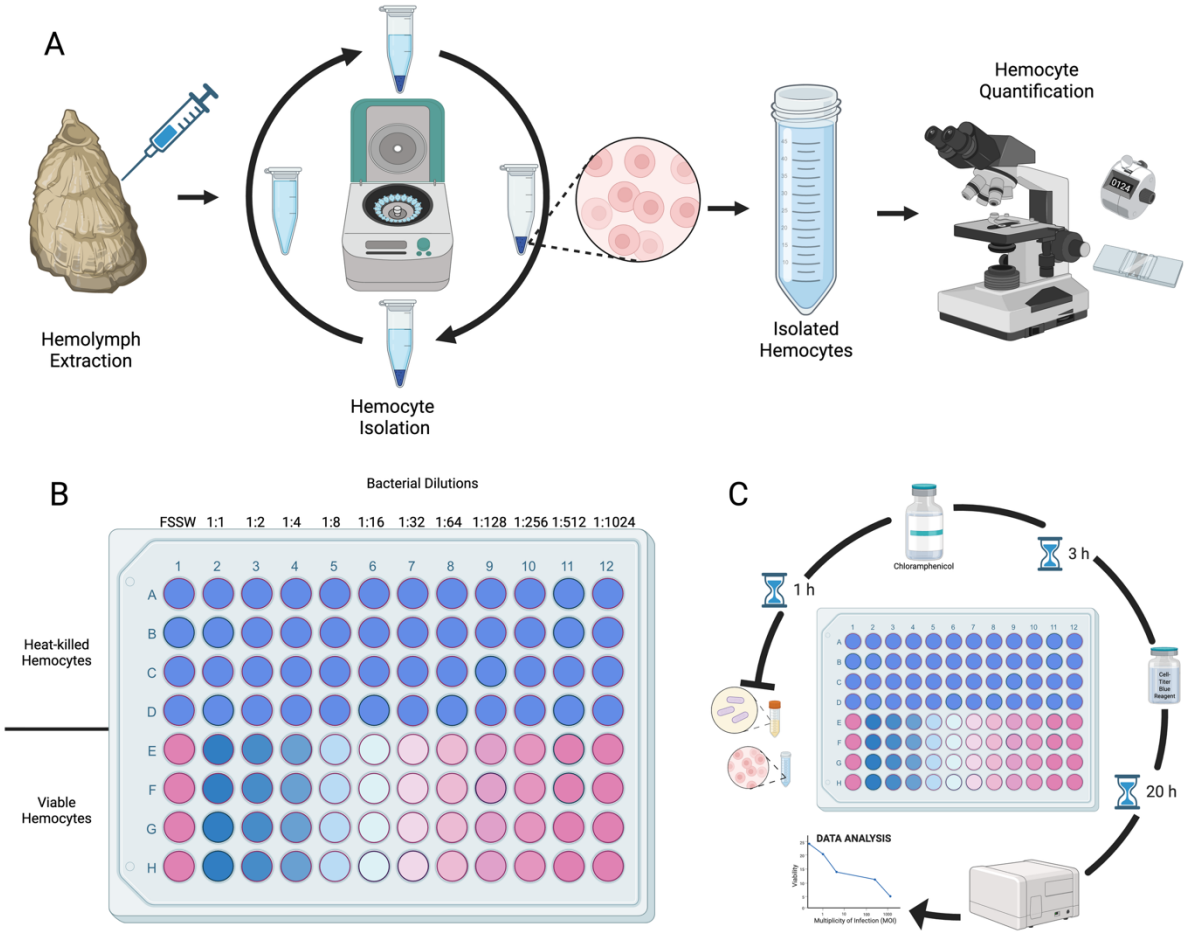


Figure 1. Overview of the hemocyte viability assay including the (A) isolation, preparation, and quantification of cell concentration; (B) plate layout, with half of the plate for the incubation of live hemocytes with bacterial dilutions, and half of the plate for heat killed hemocytes plus bacterial dilution controls; and (C) the procedure of the hemocyte viability assay using the resazurin dye. Figure created with BioRender.com.

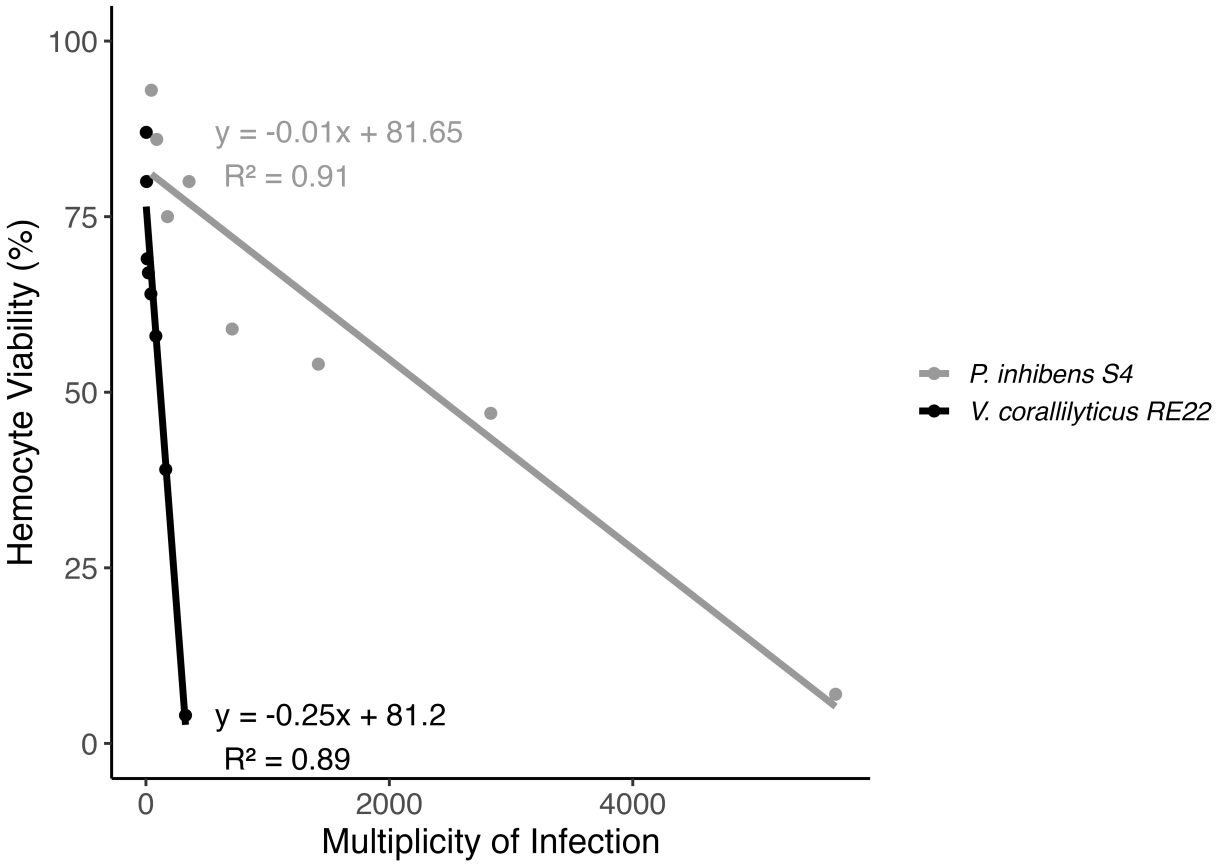
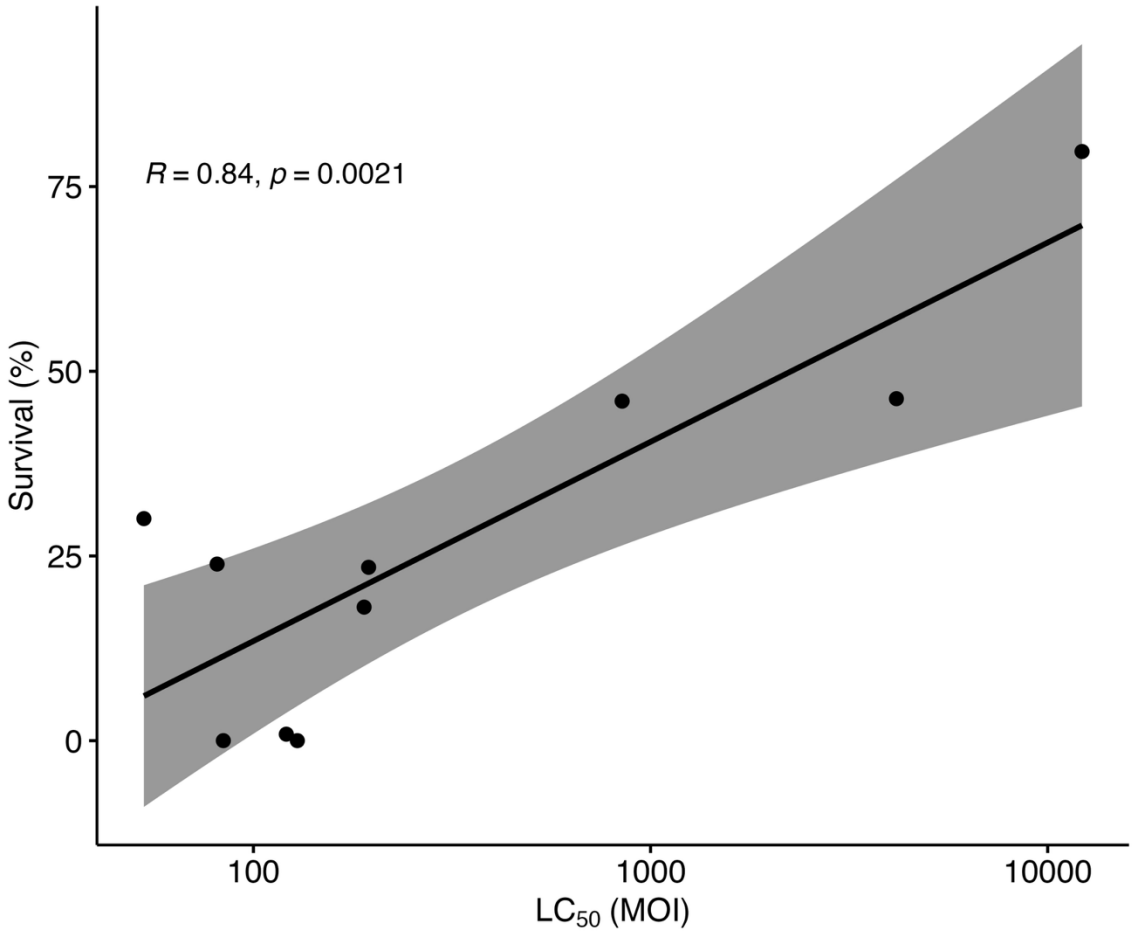


Figure 2. Relationship between multiplicity of infection (MOI) and hemocyte viability measured by resazurin reduction. Linear regression analysis of MOI and hemocyte viability for the pathogen *V. coralliilyticus* RE22 and the probiont *P. inhibens* S4 demonstrates the negative correlation between MOI and hemocyte metabolic activity, indicating reduced hemocyte viability at higher bacterial loads. Data points represent mean values from replicate wells, with fitted regression lines, corresponding equations, and R^2 values.

528



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530 Figure 3. Relationship between hemocyte and larval viability assays. Linear regression of larval
531 survival after treatment with pathogenic isolates (as defined as those causing >20% mortality) and
532 their corresponding LC_{50} values on adult oyster hemocytes based on multiplicity of infection
533 (MOI). The survival percentage is plotted against the LC_{50} values on a logarithmic scale. Larval
534 survival data points represent mean values of 3 wells of larvae treated with 10^5 CFU mL^{-1} of
535 bacteria for 16-20 hours. Pearson correlation and linear regression with 95% confidence intervals
536 are shown.