# 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, resulting 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 based on the resazurin assay was developed as an alternative for testing bacterial pathogenesis. The assay was optimized using eastern oyster hemocytes, and the lethal concentration $(LC)_{50}$ values of a well-known bacterial pathogen of larvae (*Vibrio coralliilyticus* RE22) (107 ± 22 multiplicity of infection (MOI)), a probiont (*Phaeobacter inhibens* S4) (2157 ± 167 MOI), and several bacterial isolates from shellfish hatcheries were determined. The most virulent isolates had an $LC_{50}$ ranging from 53 to 195 MOI, indicating high pathogenicity. To validate the assay, the isolates were tested on oyster larvae, confirming that bacterial isolates with low $LC_{50}$ (<200 MOI) caused significant larval mortality, while other isolates exhibiting $LC_{50}$ values ranging from 690 to 25389 MOI showed no adverse effect on the oyster larvae. This high-throughput hemocyte viability assay provides a reliable and less time-consuming alternative to larval assays, particularly during the off-season, facilitating year-round research on bacterial pathogenicity in bivalves.

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

33 **INTRODUCTION**

34 Bivalves are important in fisheries, aquaculture, and ecosystem structure and function.
35 Because of these critical roles, significant research has focused on their immunological responses
36 to infectious diseases, suggesting that they could potentially be used as a model system to study
37 human health (Fernández Robledo et al., 2019). Like other invertebrates, bivalves have a diverse
38 range of immune cells, genes, and proteins. Although mucosal tissues in contact with the
39 environment play an important role in host defense, hemocytes are the backbone of the bivalve
40 immune system (Allam and Raftos, 2015).

41 Among the various threats to bivalve health includes the bacterial pathogens, which are a
42 leading cause of disease outbreaks in bivalve larvae, resulting in significant mortality and
43 economic losses. Most bacterial diseases of bivalves are caused by a large range of *Vibrio* spp.,
44 *Pseudomonas,* and *Aeromonas* (Zannella et al., 2017). Due to the filter-feeding behavior of
45 bivalves, they concentrate a rich and diverse bacterial commensal microbiota, comprising several
46 species belonging to distinct genera including *Vibrio, Pseudomonas, Acinetobacter,*
47 *Photobacterium, Moraxella, Aeromonas, Micrococcus* and *Bacillus* (Kueh and Chan, 1985;
48 Zannella et al., 2017); some of them may be harmful in larval rearing systems but not in the wild.
49 These bacteria can rapidly proliferate in aquaculture environments, often exploiting stress or
50 environmental fluctuations to infect and overwhelm bivalve hosts. *Vibrio* spp. are particularly
51 well-characterized, producing virulence factors like hemolysins and ciliostatic toxins which cause
52 deciliation, loss of velar epithelial, and abnormal swimming behavior (Paillard et al., 2004).

53 A major challenge in studying the pathogenesis of bacterial isolates and their effects on the
54 larvae is the limited availability of healthy bivalve larvae throughout the year. This seasonal

55 limitation impedes the ability to consistently assess the pathogenicity of bacterial isolates and their
56 interaction with host organisms under varying environmental conditions. As larvae are often only
57 available during specific times of the year, researchers are left with a narrow window for
58 experimental testing, which may limit the robustness and reproducibility of findings. This
59 restriction can delay research into bacterial infections, disease management, and the development
60 of effective treatments for bivalve aquaculture. To address the challenges of studying bacterial
61 pathogenicity and virulence in bivalve larvae, alternative high-throughput methods that are more
62 consistent, accessible, repeatable, and faster are essential. The Alamar Blue assay, also known as
63 the resazurin assay, has emerged as a valuable tool for assessing hemocyte viability and metabolic
64 activity. This assay relies on the reduction of resazurin to resorufin, a fluorescent compound that
65 serves as an indicator of cellular metabolic activity (O'Brien et al., 2000). This assay has been
66 widely used to measure cell viability and cytotoxicity in a range of biological and environmental
67 systems and several cell types, including bacteria, yeast, fungi, protozoa, and cultured mammalian
68 and piscine cells (O'Brien et al., 2000; Petiti et al., 2025; Rampersad, 2012).

69 Bivalves have an open circulatory system in which hemolymph, containing hemocytes,
70 bathes all the organs before returning to the heart via sinuses and respiratory structures. These
71 circulating hemocytes are primarily responsible for defending against pathogens through
72 mechanisms such as phagocytosis and encapsulation (Canesi et al., 2002; Fisher, 1986).
73 Additionally, antibacterial effectors, opsonins, nonspecific hydrolytic enzymes, and toxic oxygen
74 intermediates found in bivalve hemolymph coordinate the immune response (Chong, 2022; Gerdol
75 et al., 2018; Song et al., 2010). The classification of bivalve hemocytes has been a subject of
76 debate, though they are generally categorized into hyalinocytes and granulocytes (de la Ballina et
77 al., 2022; López et al., 1997). The latter can be further subdivided into eosinophilic granular

78 hemocytes and basophilic granular hemocytes (López et al., 1997; Wootton et al., 2003). Other
79 hemocyte types, such as morula-like cells and blast-like cells, have also been reported in some
80 species (Donaghy et al., 2009; Wootton et al., 2003). Among these, granular hemocytes are the
81 most numerous and function as the primary phagocytic cells in bivalves(Allam et al., 2001;
82 Cajaraville and Pal, 1995). Hemocytes can recognize, locate, ingest, transport, and digest foreign
83 particles, playing a central role in phagocytosis, encapsulation, inflammation, and wound repair
84 (Fisher, 1986). Mobility is a critical characteristic of hemocytes, enabling them to migrate toward
85 pathogens and perform immune functions efficiently. Studies on *Crassostrea virginica* and
86 *Mercenaria mercenaria* have demonstrated that granulocytes are the predominant phagocytes
87 responsible for host defense (Cheng et al., 1975; Foley and Cheng, 1975). While granulocytes are
88 generally associated with pathogen clearance, some evidence suggests that agranulocytes may also
89 contribute to immune responses (Sokolnikova et al., 2023).

90 Hemocyte viability is a measure of the proportion of alive cells to evaluate the overall
91 hemocyte health. Viability assays can be assessed based on cellular metabolism, enzyme activity,
92 or cell membrane integrity. The widely used approach was double fluorescence staining with
93 membrane-permeable nuclear dyes, such as SYBR, and the membrane-impermeable dyes, such as
94 propidium iodide (PI), and detected by use of fluorescence microscopy or flow cytometry (Allam
95 et al., 2001). In contrast, the resazurin assay offers a high-throughput, plate-based method that is
96 both rapid and quantifiable.

97 The goal of this work is to develop an alternative and high-throughput viability assay that
98 can be used to assess bacterial infections and virulence without relying on the seasonal availability
99 of bivalve larvae. Hemocytes, as key immune cells in mollusks, can serve as an alternative to

100 assess the impact of bacterial infections, enabling the testing of bacterial virulence in the absence
101 of larvae. This assay can provide valuable insights into host-pathogen interactions, mechanisms of
102 pathogenesis, immune responses, and virulence quantification of bacterial isolates. This could
103 enhance the efficiency and flexibility of pathogen characterization, ultimately contributing to
104 better disease management and prevention strategies in bivalve aquaculture.

105

106 **MATERIALS AND METHODS**

107 **Bacteria Isolation and Identification**

108 Bacteria were isolated from bivalve (eastern oyster *Crassostrea virginica* and hard clam
109 *Mercenaria mercenaria*) larvae samples from different hatcheries (n = 4) in the Atlantic Coast of
110 the US (hatchery identity not provided per non-disclosure agreement with the hatcheries). Upon
111 samples receipt, larvae were washed and placed in filtered sterile seawater (FSSW) overnight at
112 room temperature (20 to 22 °C) with aeration before bacterial isolation. To isolate bacterial strains,
113 larvae were filtered through a 40-µm mesh and washed with FSSW. A 100 µL aliquot of the
114 concentrated larvae was combined with 100 µL FSSW and macerated using a sterile grinder or
115 pipette tip until the sample became homogeneous. A 100 µL aliquot was then taken for serial
116 dilution. Then, 20 µL was plated on various media, including YP30 (yeast extract (0.5 %), peptone
117 (0.1 %), sea salts (3 %) and agar (1.5%), pH 7.6) (Karim et al., 2013), marine agar
118 (MilliporeSigma, USA), seawater tryptone agar (SWT, tryptone (0.5%), yeast extract (0.3 %),
119 glycerol (0.3%), agar (1.5%), pH 7.6), and nutrient-free agar plates, and incubated for 2-5 days at
120 27 °C. After incubation, distinct colonies were selected and streaked onto fresh sterile plates until
121 single colonies were isolated.

122 The DNA from isolated bacteria were extraction following a modified protocol (Li et al.,
123 2023). Briefly, overnight culture (500 µL) of each isolate was harvested and resuspended in
124 DNase-free water with 0.1 g of 0.1 mm glass beads (Sigma-Aldrich, USA). The cells were heated
125 at 80 °C at 500 rpm for 10 minutes, followed by RNase treatment (Qiagen, Germany). Bacterial
126 isolates were identified by 16S rRNA sequencing using universal primers 27F (5'-
127 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')
128 (Weisburg et al., 1991). PCR reactions were prepared in a total volume of 25 µL, comprising 5 µL

129 of extracted DNA, 12.5 µL of PCR Mastermix, 1 µL each of forward (27F) and reverse (1492R)
130 primers, and 5.5 µL of nuclease-free water. Amplification was performed using the following
131 thermal cycling conditions: initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at
132 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds;
133 followed by a final extension at 72°C for 5 minutes. PCR products were purified using a Qiagen
134 PCR purification kit following the manufacturer's protocol and sequenced at the URI-INBRE
135 facility. Sequence identification was conducted by comparing obtained sequences to the NCBI 16S
136 database using BLAST (Camacho et al., 2009).

137 **Preparation of Bacterial Dilutions**

138 Bacterial isolates (Table 1) were cultured overnight on Yeast Peptone 30 agar plates at
139 27°C for 18-24 hours. After incubation, individual colonies were picked and transferred to liquid
140 YP30 medium, where they were incubated at 27°C with shaking (150 rpm) for 18-24 hours.
141 Following the incubation, the bacterial cultures were centrifuged at 13,000 rpm for 5 minutes to
142 pellet the cells. The supernatant was discarded, and the bacterial pellet was washed twice with
143 filtered seawater (FSSW) to remove the residual medium. The cell pellet was then resuspended in
144 FSSW, and the optical density ($OD_{600}$) was adjusted to the desired concentration ($10^8$ CFU $mL^{-1}$).

145 To prepare for the assay, a series of two-fold bacterial dilutions was made in a 96-well
146 plate. The final bacterial concentration in each well was verified by plating appropriate dilutions
147 onto YP30 agar plates and determining colony counts to confirm the accuracy of the dilution series
148 (Karim et al., 2013).

149 **Preparation of Oyster Hemocytes**

150 Adult eastern oysters (*Crassostrea virginica*) from Rhode Island farms were collected for
151 hemocyte isolation, and all procedures were conducted under aseptic conditions. The shells of the

152 oysters were scrubbed clean and rinsed in freshwater and sprayed with 70% ethanol. For each
153 oyster, a small hole was made near the adductor muscle using flame-sterilized pliers. Then, a sterile
154 1.5" 21 G needle, attached to a 3 mL syringe, was primed with 100 µL of ice-cold, filtered sterile
155 seawater (FSSW, salinity of 28 psu), ensuring that air bubbles were removed from the syringe.
156 The primed needle was inserted into the adductor muscle notch, and approximately 1 mL of
157 hemolymph was extracted from each oyster. The needle was rotated gently during extraction to
158 help remove tissue adhering to the needle. Hemolymph samples were passed through a 40 µm
159 mesh filter into a 50 mL Falcon tube kept on ice to remove large tissue debris.

160 The pooled hemolymph (6-12 oysters) was then centrifuged at 2500 rpm for 3 minutes at
161 4°C to collect the hemocytes. The supernatant was discarded, and the hemocytes were washed
162 twice with FSSW and resuspended in the same solution. Hemocyte concentration and viability
163 were evaluated using a hemocytometer, and concentration was adjusted to between $1.0 \times 10^6$ and
164 $2.0 \times 10^6$ cells $mL^{-1}$. For heat-killed hemocyte preparation, half of the total hemocyte suspension
165 volume was placed in a water bath at 90°C for 10 minutes (Fig. 1A). Live and heat-killed (HK)
166 hemocyte solutions were kept on ice for use in subsequent assays.

167 **Hemocyte Viability Assay Development**

168 To assess the impact of bacterial pathogens and probiotics on hemocytes, we developed a
169 hemocyte viability assay based on the reduction of resazurin dye. This approach quantifies the
170 hemocyte metabolic activity as a proxy for cell viability following bacterial exposure. The assay
171 workflow is illustrated in Fig. 1, outlining the key steps: collection and preparation of alive and
172 heat-killed hemocytes, bacterial serial dilutions, bacterial-hemocyte interaction, antibiotic
173 addition, a 20-hr incubation period, and finally, measurement of cellular metabolic activity via
174 fluorescence.

175 The hemocyte viability was assessed resazurin-based dye CellTiter-Blue™ (Promega). The
176 assay was carefully optimized to determine the appropriate hemocyte concentration, antibiotic
177 concentration, and incubation durations for each phase: hemocyte–bacteria interaction, antibiotic
178 treatment, and resazurin metabolism. For the assay, black clear-bottom 96-well plates (Corning,
179 USA) were used. Hemocytes Fifty microliters of either live and heat-killed hemocytes
180 (background control) were added to designated wells of the plate (Fig.1B). A known concentration
181 of pathogenic *Vibrio coralliilyticus* RE22 (Estes et al., 2004) and probiotic *Phaeobacter inhibens*
182 S4 (Karim et al., 2013) were prepared by dilution and spot plating to determine the actual bacterial
183 concentration required for calculating the multiplicity of infection (MOI), defined as the ratio of
184 infectious agents (e.g., bacteria or viruses) to target host cells. Fifty microliters of the bacterial
185 suspension were added to each well containing the hemocytes, each replicated four times. The
186 plates were incubated for 1 hour at room temperature to allow bacterial interaction with hemocytes.
187 After incubation, chloramphenicol was added to the wells to a final concentration of 200
188 $\mu g\ mL^{-1}$ to prevent further bacterial proliferation during the assay. The plates were incubated for
189 an additional 3 hours. Next, 10 µL of resazurin reagent was added to each well, and the plate was
190 shaken gently for 10 seconds. The plate was then incubated for 20 hours at room temperature to
191 allow the reduction of resazurin. After the incubation period, the plate was shaken again for 10
192 seconds, and fluorescence was measured using a fluorometer (BioTek, USA) at an excitation
193 wavelength of 560 nm and an emission wavelength of 590 nm (Fig. 1C).

194 **Data Calculation and Analysis**

195 To calculate hemocyte viability, fluorescence from heat-killed hemocyte wells was
196 subtracted from the fluorescence readings of live hemocyte wells (no treatment control) to account
197 for background fluorescence. The heat-killed hemocyte control consists of hemocytes that have

198 been inactivated by heat treatment, rendering them metabolically inactive. This control helps
199 account for background fluorescence as well as any potential effects caused by bacteria utilizing
200 nutrients released from dead hemocytes, which could influence bacterial metabolism and interfere
201 with assay readings. The relative viability of the hemocytes was determined by normalizing the
202 fluorescence of the treated hemocyte samples to that of the control wells. Relative viability was
203 calculated using the following formula:

204 $$\text{Viability (\%)} = \frac{F_{Experimental} - F_{Blank}}{F_{Control} - F_{Background}} \times 100$$

205 Where:

206 $F_{Experimental}$ = fluorescence reading of the well containing hemocytes and the pathogen

207 $F_{Blank}$ = fluorescence reading of the well containing heat-killed hemocytes and the pathogen

208 $F_{Control}$ = fluorescence reading of the well containing the hemocytes without the pathogen

209 $F_{Background}$ = fluorescence reading of the well containing the heat-killed hemocytes without the
210 pathogen

211

212 The MOI was calculated by dividing the bacterial concentration (CFU $mL^{-1}$) by the
213 hemocyte concentration (cells $mL^{-1}$). Then, a linear regression analysis was performed to plot the
214 relationship between MOI and relative viability. Using the regression equation, the lethal
215 concentration for 50% viability ($LC_{50}$) was determined.

216

217 **Larval Assay**

218 To compare the result of the hemocyte viability assay with the larval assay, the effect of
219 the bacterial isolates on larval viability was assessed using a previously described protocol
220 (Gómez-León et al., 2008) with modifications. Briefly, 6-8-day-old healthy eastern oyster and

221 hard clam larvae (veligers) were added to 24-well plates at 75-100 larvae $mL^{-1}$. Then, bacterial
222 isolates were added at a final concentration of $10^5$ CFU $mL^{-1}$ and incubated for 16-20 h at room
223 temperature. At this point, larval mortality was evaluated by visual examination under a
224 microscope (Leica, 400x magnification). Larvae mortality was calculated as follows:
225

226 $$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of dead larvae} + \text{Number of alive larvae}} \times 100$$

227

228 **RESULTS**

229 **Assay Optimization**

230 To eliminate bacterial growth during the incubation with the resazurin dye, antibiotics
231 (chloramphenicol) were added to the wells after hemocytes were incubated with the live bacteria
232 to allow for bacterial effect on live hemocytes. Different concentrations of chloramphenicol were
233 added in wells of *V. coralliilyticus* RE22 ($10^8$ CFU $mL^{-1}$) and hemocytes ($10^6$ cells $mL^{-1}$),
234 respectively, to determine the minimum antibiotic concentration that affects the growth of the
235 bacteria but not the viability of the hemocytes. The minimum concentration of chloramphenicol
236 that significantly decreased or inhibited the growth of *V. coralliilyticus* RE22 was 200 µg $mL^{-1}$.
237 On the other hand, hemocyte viability remained even at higher antibiotic concentrations (Table 2).
238 This approach helps prevent bacterial proliferation, which could otherwise artificially increase the
239 reduction rate of the dye. It was determined that a hemocyte density of 1 to 2 × $10^6$ cells/mL is
240 optimal for reliable assay performance. Furthermore, fluorescence readings stabilized after 20
241 hours of incubation with the resazurin dye, indicating sufficient time for metabolic conversion and
242 accurate viability measurement. Overall, the assay lasts for 24 h, including the 1 h bacteria-
243 hemocyte interaction and 3 h antibiotic treatment (Fig. 1C).

244 **Hemocyte Viability Assay**

245 To test the capability of the assay in quantifying bacterial virulence, well-known
246 pathogenic (*V. coralliilyticus* RE22) and probiotic (*Phaeobacter inhibens* S4) bacteria were used.
247 A known pathogenic isolate, *V. coralliilyticus* RE22, had a computed $LC_{50}$ value of 107 ± 22,
248 while the probiotic *P. inhibens* S4 had 2157 ± 167 (Fig. 2). The assay was repeated three times to
249 get the standard deviation. The coefficient of variation was calculated for inter assay accuracy and
250 has 7.7% and 20.5% for S4 and RE22.

251 The assay was further applied to evaluate the virulence of several bacterial isolates newly
252 isolated from oyster and clam larvae. (Table 1). The $LC_{50}$ values of the bacterial isolates ranged
253 from 53 to 25,389 MOI (Table 3). Among these, the most virulent isolate to oyster larvae was *V.*
254 *toranzoniae* CH7, with an $LC_{50}$ of 53 MOI, followed by *V. rotiferianus* CH3 (81 MOI), *V. fortis*
255 CH6 (86 MOI), *V. neptunius* DEN11 (129 MOI), *V. chagasii* CH4 (190 MOI), and *C. lytica* CH30
256 (195 MOI). In contrast, the highest $LC_{50}$ values among the isolates corresponded to *V. chagasii*
257 CLAM14 (25,389 MOI) and *T. ascidiaceicola* CLAM15 (20,315 MOI), suggesting these are non-
258 pathogenic isolates on oyster.

259 **Larval Assay**

260 To confirm the result of the hemocyte viability assay, the tested isolates were used to
261 challenge eastern oyster and hard clam larvae for 16 – 20 h at a standardized concentration of $10^5$
262 CFU $mL^{-1}$ (Fig. 3) . Oyster larvae challenged with the known pathogen *V. coralliilyticus* RE22
263 had almost 100% mortality. Similarly, potential pathogenic isolates *V. fortis* CH6 and *V. neptunius*
264 DEN11 also had 100% mortality. In addition, *V. chagasii* CH4, *C. lytica* CH30, *V. rotiferianus*
265 CH3, *V. toranzoniae* CH7, and *V. tubiashii* DEN41 had larval survival rates below 50%,
266 confirming their pathogenicity towards the larvae. Conversely, larvae treated with the potential

267 probiotic isolates exhibited a relative survival rate of 96-100%, suggesting these isolates are
268 generally non-pathogenic and safe for larvae. In general, bacterial isolates that showed $LC_{50}$ values
269 below 200 MOI (*i.e., V. toranzoniae* CH7, *V. rotiferianus* CH3, *V. fortis* CH6, *V. neptunius*
270 DEN11, *V. chagasii* CH4, and *C. lytica* CH30) demonstrated significant pathogenicity towards
271 oyster larvae.

272 In clam larvae, highest mortality was observed in *V. coralliilyticus* RE22, *V. tubiashii*
273 (DEN41 and DEN12), and *V. neptunius* DEN11. Two isolates *V. chagasii* CLAM14, *T.*
274 *ascidiaceicola* CLAM15 that did not cause significant mortality in oyster larvae, exhibited notably
275 higher mortality rates in the clam larvae from which they were originally isolated.

276

277 **DISCUSSION**

278 The hemocyte viability assay offers a rapid, high-throughput alternative to traditional larval
279 assays for assessing bacterial pathogenicity in larvae. Unlike larval-based methods, which are
280 limited by the seasonal availability of healthy larvae and can be time-consuming and variable, this
281 assay provides a consistent and quantifiable measure of bacterial effects on hemocytes. To our
282 knowledge, this is the first report of using resazurin-based viability measurement in bivalve
283 hemocytes to test bacterial pathogenicity. By using resazurin reduction as a measure for hemocyte
284 viability, the assay enables sensitive detection of pathogenic impacts in a controlled, reproducible
285 format. One major challenge encountered during the assay development was the potential
286 interference of bacterial growth with the resazurin, as metabolically active bacteria can also reduce
287 the dye, confounding results. This issue was addressed by the addition of antibiotics after initial
288 bacterial-hemocyte interaction, effectively halting bacterial proliferation during the assay period
289 and ensuring fluorescence readings primarily reflect hemocyte viability. We found that hemocytes

290 remain capable of reducing resazurin even in the presence of antibiotics, allowing effective
291 inhibition of bacterial growth during incubation without compromising the assay. Similar to
292 previous reports, hemocyte viability was not affected by the addition of antibiotics (Lacaze et al.,
293 2015; Matozzo et al., 2016).

294 Potential limitations include the fact that this in vitro assay may not fully capture the
295 complexity of whole-organism responses in larvae, such as tissue-specific effects or systemic
296 immune responses. Additionally, some bacterial species may exhibit variable sensitivity to
297 antibiotics or resazurin, potentially affecting assay accuracy, thus other antibiotics or even
298 antibiotic cocktail can be optimized for future use. Also, the use of hemocytes from other bivalves
299 can be explored to test the host specificity of bacterial isolates being tested. Nonetheless, the assay
300 represents a valuable tool for rapid screening of bacterial virulence and host-pathogen interactions,
301 complementing more traditional larval infection studies.

302 ***Hemocyte viability assay corresponds with the larval assay***

303 In this study, we assess the result of the hemocyte assay using the same test isolates we
304 tested on oyster larvae. To evaluate the pathogenicity, a 20% mortality threshold was arbitrarily
305 set to identify the pathogenic isolates. The correlation between mortality and $LC_{50}$ of the
306 pathogenic isolates was strong, with a Pearson correlation coefficient of 0.84 ($P$ = 0.0022),
307 indicating a significant relationship (Fig. 4). Similar to the results of the hemocyte assay, *V.*
308 *toranzoniae* CH7, *V. rotiferianus* CH3, *V. fortis* CH6, *V. neptunius* DEN11, *V. chagasii* CH4, and
309 *C. lytica* CH30, which showed less than 200 MOI $LC_{50}$ values, caused more than 20% mortality
310 in the oyster larvae. In addition, *V. tubiashii* (DEN12 and DEN41) and *V. alginolyticus* DEN30
311 also had significant mortality on the larvae, although they showed higher $LC_{50}$. The tested potential

312 pathogens *V. tasmaniensis* CLAM18*, V. cyclitrophicus* CLAM19*, V. chagasii* CLAM14, *T.*
313 *ascidiaceicola* CLAM15, and *V. alginolyticus* DEN30, which showed high $LC_{50}$ values, did not
314 affect the larval survival significantly. It is important to note that four of these isolates, except the
315 latter, were isolated from clams; and when tested against clam larvae (*M. mercenaria*), they
316 resulted in mortalities of 47%, 17%, 31%, and 51%, respectively. This host-specific pathogenicity
317 is a well-known occurrence of bacterial pathogens. Because different hosts occupy diverse
318 environmental niches, a pathogen's fitness can vary depending on the host species. Pathogens that
319 are well-adapted to certain hosts can evolve into unique strains, a phenomenon known as
320 polymorphism. Alternatively, certain pathogens may evolve into generalists capable of surviving
321 and replicating in multiple host species via a variety of methods, a process known as polyphenism
322 (Bäumler and Fang, 2013).

323 ***Pathogenic bacteria require less concentration to cause hemocyte mortality***

324 In this study, several pathogenic bacterial isolates (*i.e., V. toranzoniae* CH7, *V. rotiferianus*
325 CH3, *V. fortis* CH6, *V. neptunius* DEN11, *V. chagasii* CH4, and *C. lytica* CH30) showed $LC_{50}$
326 values below 200 MOI. This indicates that these isolates might produce toxins that degrade the
327 hemocytes. These results are consistent with previous reports indicating pathogenicity for these
328 species. *V. toranzoniae* and several other *Vibrio* spp. (*V. lentus*, *V. crassostreae*, and *V.*
329 *cyclitrophicus*) caused significantly higher hemocyte cytotoxic rates in the scallop, *Argopecten*
330 *purpuratus* (González et al., 2025). Similarly, *V. rotiferianus* has also been reported to show
331 pathogenicity in insect infection models (Harrison et al., 2022). *V. fortis* has been implicated in
332 the heat stress-induced mortality of *C. gigas* (Green et al., 2019). *V. neptunius* is a natural part of
333 the mollusk microbiota and an opportunistic pathogen that causes disease outbreaks in oysters and

334 clams. It is known to produce siderophores, piscibactin, and amphibactin, which contribute
335 significantly to its virulence (Galvis et al., 2021). In addition, *V. chagasii* was pointed out as the
336 causative agent of the massive larval mortalities of the farmed scallop (*A. purpuratus*) in Chile
337 (Urtubia et al., 2023). *C. lytica* has been isolated in the eroded mantle epithelium of juvenile paua
338 (*Haliotis iris*) in New Zealand during an outbreak of bacterial disease of the epithelium of the
339 mantle, epipodium, and foot (Diggles, 2005).

340 Other *Vibrio* strains tested, including *V. tubiashii* DEN12, DEN41, *V. tasmaniensis*
341 CLAM18*, V. cyclitrophicus* CLAM19*, V. chagasii* CLAM14, and *V. alginolyticus* DEN30,
342 showed high $LC_{50,}$ indicating low to non-pathogenicity for hemocytes. Vibrios are gram-negative,
343 curved rod-shaped, motile, heterotrophic, rapid-growing, and highly adaptable bacteria comprising
344 more than 140 species that are ubiquitous in aquatic environments. Most are non-pathogenic. They
345 are often associated with phytoplankton, zooplankton, crustaceans, and bivalves, showing free-
346 living, symbiotic, and, in some cases, pathogenic life strategies. Pathogenicity is determined at the
347 strain level, with most species exhibiting a diversity of nonpathogenic and pathogenic strains
348 (Gómez-Chiarri et al., 2025). Furthermore, pathogenicity depends on the host species, their life
349 stage, the number of bacteria, and environmental factors (Zannella et al., 2017).

350 **CONCLUSIONS**

351 This study aimed to develop an alternative assay for assessing bacterial pathogenicity in
352 bivalves, utilizing adult oyster hemocytes as a substitute for oyster larvae to overcome the issue of
353 larval shortages during the off-season. The results demonstrated that the developed hemocyte
354 viability assay is an effective tool for testing the pathogenicity of bacterial isolates in oysters.
355 Importantly, this assay can be further adapted to assess the pathogenicity of bacterial isolates in

356 hemocytes from other bivalve species, such as clams, scallops, and mussels. This flexibility makes
357 it a valuable tool for investigating host-specific pathogens across a broader range of bivalve
358 species.

359 **Conflict of Interests**

360 The authors declare that they have no competing interests

361

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371

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

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

502
503

504 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 | - | + |

505 + = resazurin dye turned into pinkish
506 - = resazurin dye remained bluish
507

508

509 Table 3. Computed $LC_{50}$ value of bacteria isolated from bivalves using the developed hemocyte
510 viability assay.

| ISOLATE TESTED | $LC_{50}$ (MOI) |
|---|---|
| *Phaeobacter inhibens* S4 | 2157 ± 167 |
| *Vibrio coralliilyticus* RE22 | 107 ± 22 |
| *Vibrio toranzoniae* CH7 | 53 |
| *Vibrio rotiferianus* CH3 | 81 |
| *Vibrio fortis* CH6 | 84 |
| *Vibrio neptunius* DEN11 | 129 |
| *Vibrio chagasii* CH4 | 190 |
| *Cellulophaga lytica* CH30 | 195 |
| *Vibrio tubiashii* DEN41 | 848 |
| *Vibrio tasmaniensis* CLAM18 | 877 |
| *Vibrio cyclitrophicus* CLAM19 | 927 |
| *Vibrio tubiashii* DEN12 | 4160 |
| *Vibrio alginolyticus* DEN30 | 12204 |
| *Tenacibaculum ascidiaceicola* CLAM15 | 20315 |
| *Vibrio chagasii* CLAM14 | 25389 |
| *Pseudooceanicola nitratireducens* NEH7 | 690 |
| *Marinomonas gallaica* CLAM9 | 704 |
| *Algoriphagus yeomjeoni* DEN5 | 935 |
| *Glutamicibacter soli* CLAM16 | 1913 |

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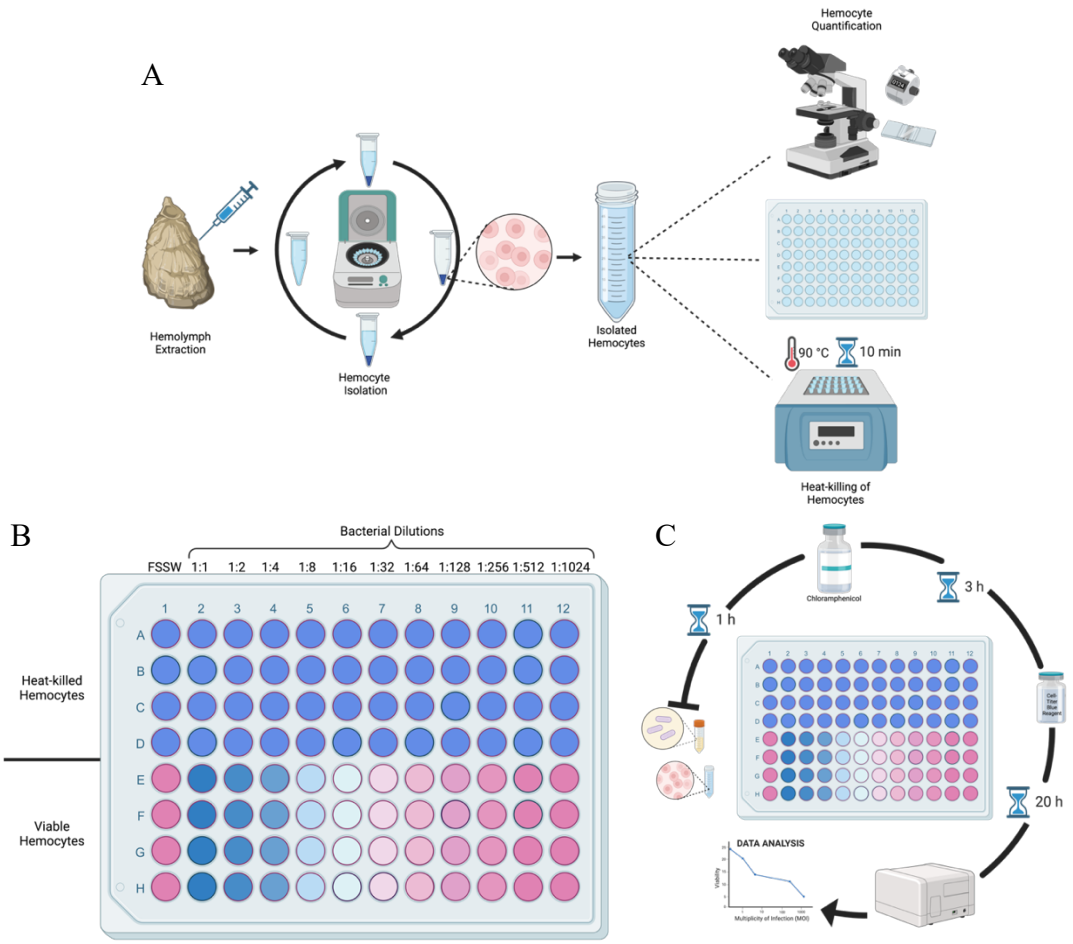



515 **Figure 1.** Overview of the hemocyte viability assay including the (A) isolation, preparation, and

516 quantification; (B) plate layout; and (C) the procedure of the hemocyte viability assay using the

517 resazurin dye. Figure created with BioRender.com.

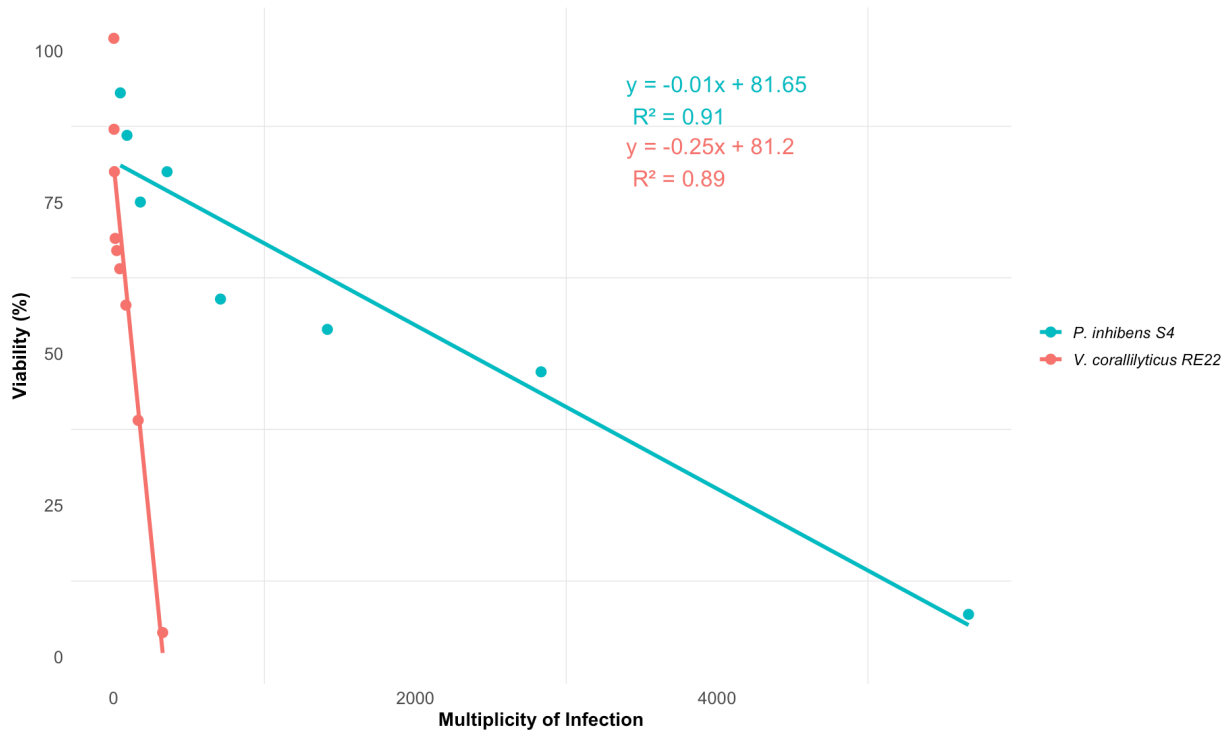
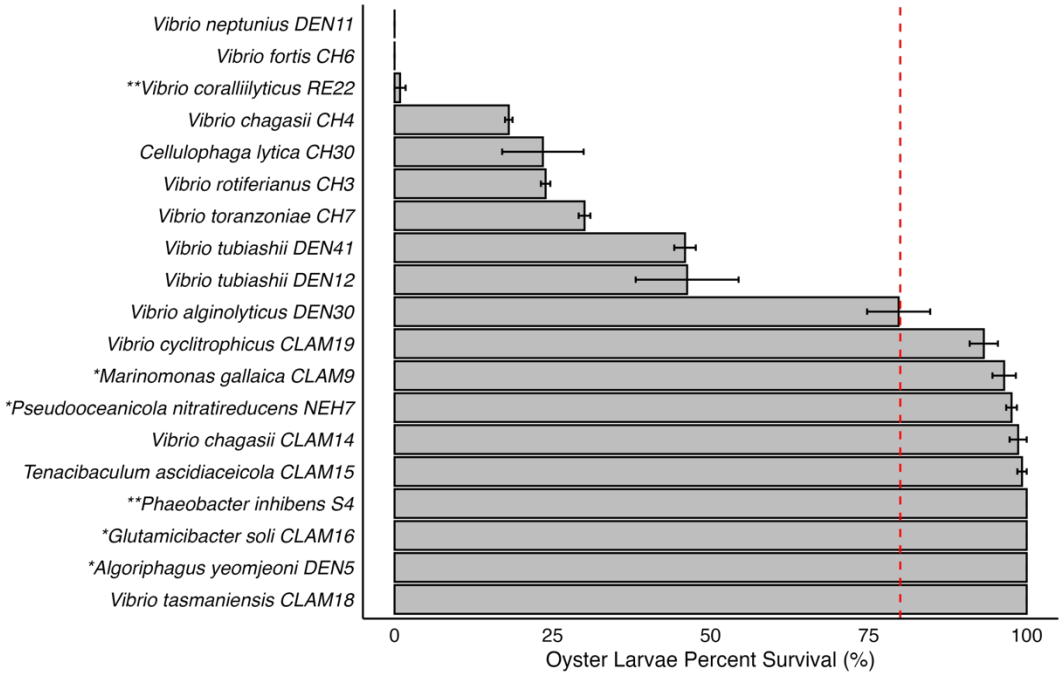



**Figure 2.** Representative example of linear regression analysis of *V. coralliilyticus* RE22 and *P. inhibens* S4. Relationship between multiplicity of infection (MOI) and hemocyte viability measured by resazurin reduction. Linear regression analysis demonstrates the negative correlation between increasing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



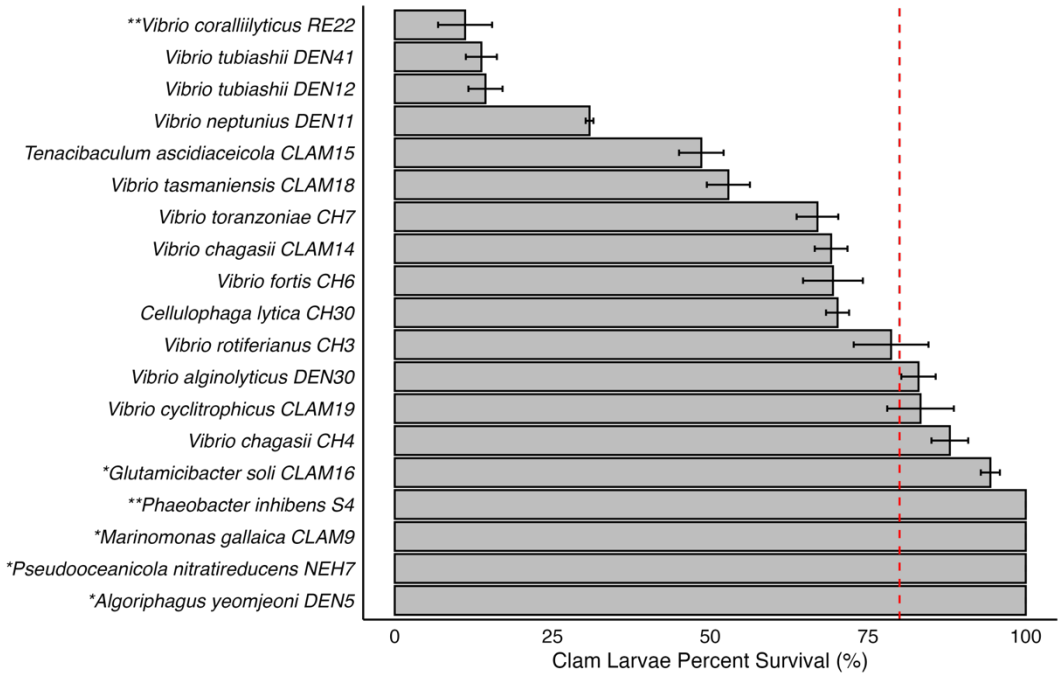
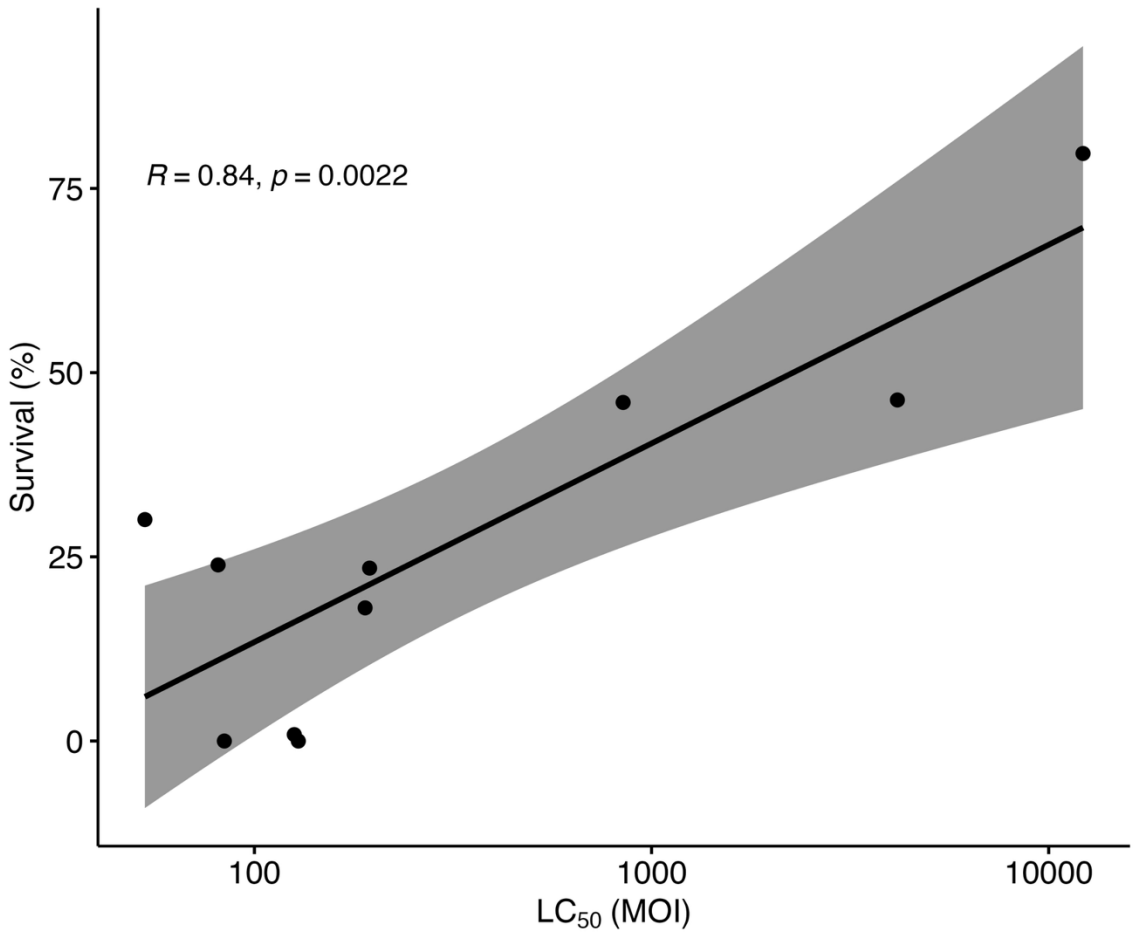




**Figure 3.** Relative percent survival of eastern oyster (upper) and hard clam (lower) larvae challenge and treated with pathogenic and probiotic isolates. Data are presented as mean ± standard error of the mean (SEM). Red dash line indicates 80% survival threshold. ** Known pathogen and probiotics. *Potential probiotics.




533

534 **Figure 4**. Correlation of the survival data from pathogenic isolates causing >20% mortalities and
535 their corresponding $LC_{50}$ values based on multiplicity of infection (MOI). The survival percentage
536 is plotted against the $LC_{50}$ values on a logarithmic scale. Survival data points represent mean
537 values. Pearson correlation and linear regression with 95% confidence intervals are shown.

538