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1 # A marine probiotic treatment against the bacterial pathogen *Vibrio* 2 *coralliilyticus* to improve the performance of Pacific (*Crassostrea gigas*) and 3 Kumamoto (*C. sikamea*) oyster larvae

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16 # Highlights

17 - A novel treatment of three combined marine probiotic bacterial isolates improved acute
18 survival of early Pacific oyster larvae exposed to a lethal concentration of the highly
19 virulent *V. coralliilyticus* strain RE22.
20 - A single dose of combined probiotics added to one-day-old Pacific oyster larvae
21 improved their subsequent growth at 14 days post egg-fertilization.
22 - Metamorphosis success was increased in two stocks of the Pacific oyster, *Crassostrea*
23 *gigas*, as well as the Kumamoto oyster, *C. sikamea*, after a single dose of the combined
24 probiotics was added to one-day-old larvae.

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27 # Abstract

28 Oyster larvae reared in hatcheries on the U.S. West coast often experience severe *Vibrio*
29 *coralliilyticus*-related mortalities early in their development. Current treatment options for
30 these molluscs are not available or feasible; however, for decades, probiotics have been
31 successfully used in finfish and crustacean shellfish culture. Consequently, the objectives of this
32 work were to 1) isolate marine bacteria from oysters and evaluate their protective activity
33 against *Vibrio coralliilyticus* infection of Pacific oyster (*Crassostrea gigas*) larvae, and 2) to
34 determine the long-term effects of probiotic additions on growth and metamorphosis of larval
35 Pacific and Kumamoto oysters (*C. sikamea*). A combination of three probiotic strains applied
36 once 24 hours post-fertilization was more effective in improving survival of larval *C. gigas*
37 exposed to lethal concentrations of *V. coralliilyticus* strain RE22, compared with separate
38 additions of individual probiotics. In addition, a single application of the probiotic combination
39 to one-day-old larvae increased the larval metamorphosis success of *C. sikamea* and both the
40 Midori and Myiagi stocks of *C. gigas*. These results suggest that probiotics are effective at
41 preventing disease and can significantly improve performance of oyster larvae, using a single
42 application early in their development.
43
44

45 Keywords: Probiotic; oyster larvae; *Crassostrea gigas*; *Crassostrea sikamea*; *Vibrio coralliilyticus*;
46 survival; metamorphosis

47

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48 # 1. Introduction

49 In the USA, oyster farms are largely dependent on high-quality seed ("eyed" larvae) from oyster
50 hatcheries. Oyster hatcheries, however, have periodically experienced severe larval losses
51 during the past two decades, leading to seed shortages and supply disruptions (Elston et al.,
52 2008; Richards et al., 2015). Initially, these losses were mainly attributed to ocean acidification
53 (Barton et al., 2012; Gray et al., 2022), and currently many hatcheries employ sophisticated
54 systems that measure and correct acidified incoming water. Unfortunately, these treatments
55 have not entirely resolved the problem, suggesting other factors, such as pathogens, likely play
56 a significant role in these losses and contribute to various sub-lethal effects on oyster health
57 (Marques et al., 2006).
58
59 Bacteria from the genus *Vibrio* are omnipresent in marine and brackish waters as commensals,
60 mutualists, or pathogens (Takemura et al., 2014). They are highly adaptable to changing ocean
61 conditions, including increasing temperature, lower pH, and salinity, and can make up more
62 than 50% of all detectable microbes during favorable conditions (Gilbert et al., 2012; Oh et al.,
63 2009; Vezzulli et al., 2010). *Vibrio coralliilyticus* has been linked to massive die-offs of Pacific
64 oyster (*Crassostrea gigas*) larvae in U.S. West coast hatcheries (Elston et al., 2008; Estes et al.,
65 2004; Richards et al., 2015) and, occasionally, mortalities in Eastern oysters cultured in U.S. East
66 coast hatcheries (Kehlet-Delgado et al., 2017). In addition to Pacific and Eastern oysters, this
67 pathogen affects commercially important Kumamoto oysters (*C. sikamea*), greenshell mussels
68 (*Perna canaliculus*), and geoduck clams (*Panopea generosa*) (Elston et al., 2008; Estes et al.,
69 2004; Kesarcodi-Watson et al., 2009; Richards et al., 2015). Furthermore, pathogenic *V.*

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70 *coralliilyticus* has been identified globally as a deadly pathogen in various marine species,
71 including finfish, corals, and several bivalve species (Alves Jr et al., 2009; Austin et al., 2005;
72 Jeffries, 1982; Kim et al., 2020).

73 Antibiotic interventions against bacterial infections in aquaculture are either not available or
74 restricted due to the risks of promoting widespread anti-microbial resistance in bacterial
75 populations (Cabello et al., 2013; Kesarcodi-Watson et al., 2008); furthermore, prophylactic
76 treatments, such as vaccines and probiotics, are either not feasible or not yet commercially
77 available for molluscan aquaculture (Pérez-Sánchez et al., 2018). This is in contrast to the many
78 probiotic products used in crustacean and finfish aquaculture (reviewed by El-Saadony et al.,
79 2021).

80 The definition of probiotics has been expanded in aquatic environments to reflect that they are
81 not solely antagonists of pathogens, but also can interact positively with both the environment
82 and their hosts. Verschuere et al., (Verschuere et al., 2000) for example, state ”[…] a probiotic is
83 defined as a live microbial adjunct which has a beneficial effect on the host by modifying the
84 host-associated or ambient microbial community, by ensuring improved use of the feed or
85 enhancing its nutritional value, by enhancing the host response towards disease, or by
86 improving the quality of its ambient environment”.

87 Included in this definition are probiotic-induced modifications of the microbial community that
88 affect host life stages known to be influenced by microbial cues. Bivalves undergo
89 metamorphosis and settlement, where they permanently transition from planktonic larvae to
90 sessile juveniles referred to as spat. Metamorphosis often results in high mortality (Durland et

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91 al., 2019; Jorquera et al., 2001). Some biofilms (Campbell et al., 2011; Devakie & Ali, 2002;
92 Rodriguez-Perez et al., 2019; Tritar et al., 1992; Wieczorek & Todd, 1998; Zhao et al., 2003),
93 individually added bacterial isolates (Freckelton et al., 2017), and bacterial supernatants (W. K.
94 Fitt et al., 1990; William K. Fitt et al., 1989; Walch et al., 1999) improve larval oyster
95 metamorphosis, while others result in inhibitory effects (Devakie & Ali, 2002; Dobretsov et al.,
96 2006). Therefore, studies on the use of probiotics in bivalve aquaculture should include their
97 effects on larval settlement and metamorphosis.

98 This study aimed to develop a combination of beneficial probiotic bacterial isolates that
99 reduced acute mortalities of early larvae of Pacific oysters (*Crassostrea gigas*) resulting from
100 exposure to pathogenic *V. coralliilyticus* strain RE22. We further evaluated whether single or
101 repeated probiotic additions resulted in longer-term benefits to larval growth and
102 metamorphosis of Pacific oysters. Lastly, we determined the effect of a single dose of the
103 combined probiotics on metamorphosis of the Miyagi and Midori strains of the Pacific oyster as
104 well as the Kumamoto oyster (*Crassostrea sikamea*).

105

106 # 2. Methods

107 ## 2.1. Isolation and initial screening of probiotic candidates

108 All experiments were conducted at Oregon State University's facilities, including the research
109 hatchery at the Hatfield Marine Science Center (HMSC) in Newport, Oregon, USA. Over 311
110 bacterial isolates were collected from water samples, microalgae tanks, oyster feces, mantle,
111 gills, the gastrointestinal tracts of healthy adult bloodstock and cultures of juvenile spat and

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112 larvae of Pacific oysters (*C. gigas*) from the HMSC research hatchery. Additional samples also
113 originated from a commercial hatchery in Netarts Bay, Oregon, and an oyster farm in Yaquina
114 Bay, Oregon. The final probiotic candidates originated from the following samples: *C. gigas* spat
115 that survived a naturally occurring mortality event (D16 and DM14) and gastrointestinal swabs
116 from adult *C. gigas* (B1 and B11). Isolates were enriched in culture broth before being streaked
117 on agar plates of modified, seawater-based Luria-Bertani agar (LBSw; 10 g of tryptone, 5 g of
118 yeast extract, and 15 g agar per liter of filtered seawater) and incubated at 25 °C for at least 24
119 hours. Plated colonies selected for further screening were grown in LBSw broth (10 g of
120 tryptone, 5 g of yeast extract per liter of filtered seawater) and agitated at 25 °C and 40 RPM on
121 a roller drum (New Brunswick TC-7; New Brunswick Scientific, USA) for 16 to 24 hours. Isolates
122 were cryopreserved in 15% glycerol and stored at -80 °C until further use. All seawater used for
123 bacterial culture was filtered (1 µm, Pentair, Golden Valley, MN) and autoclaved.
124
125 Isolates were first screened for their ability to inhibit the growth of *V. coralliilyticus* strains RE22
126 (Richards et al., 2021) and RE98 (Richards et al., 2014) on LBSw agar plates. Freezer stocks were
127 re-streaked onto LBSw-agar plates and incubated at 25 °C for 24 hours. One colony of each
128 candidate was inoculated into LBSw broth and grown with agitation for at least 24 hours. Five µl
129 of the overnight candidate culture were then dropped onto fresh lawns of approximately 10^4
130 CFU of *V. coralliilyticus* RE22 and/or RE98. These 10 cm-diameter LBSw-agar plates were
131 incubated overnight at 25 °C before being checked for zones of inhibition between the probiotic
132 candidates and *V. coralliilyticus*. In addition, putative probiotic candidates were screened on
133 *Vibrio*-selective Thiosulfate Citrate Bile Salt Sucrose (TCBS; Becton Dickinson or Sigma-Aldrich,

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134 USA) agar to identify and exclude *Vibrio* spp. during this initial screening process (project
135 workflow in S1 Figure).
136

137 2.2. Culture of probiotic bacterial isolates and the pathogen *Vibrio* 138 *coralliilyticus* strain RE22 for use in oyster larval assays

139 Fresh cultures of bacteria were prepared from cryopreserved samples for every oyster larval
140 experiment. *V. coralliilyticus* strain RE22 was used for all experiments in this study. This strain
141 was isolated from a commercial shellfish hatchery in the Pacific Northwest and is highly
142 pathogenic, often resulting in larval mortalities of 100% at 26 °C (Estes et al., 2004). All
143 probiotic candidates that remained in the evaluation after the initial screening were grown as
144 described above. The bacteria were then washed twice via centrifugation at 3900 x g,
145 resuspended in autoclaved seawater, and the optical density (OD) measured at 600 nm with a
146 spectrometer (Beckman DU 530). The cultures were then diluted with autoclaved seawater to a
147 final concentration in the larval culture water of 6 x 10^3 CFU/mL for *V. coralliilyticus* and 1 x 10^4
148 CFU/mL for each probiotic candidate unless otherwise specified.
149

150 2.3. Production of Pacific oyster (*C. gigas*) and Kumamoto oyster (*C.* 151 *sikamea*) D-larvae

152 Oyster broodstock was conditioned and strip-spawned to collect gametes (Langdon et al.,
153 2003). The eggs were fertilized and incubated at a density of 50 to 100 eggs/mL in 30 L
154 containers filled with seawater at 25 °C, 32 ± 2 ppt salinity, and pH of 8.2 ± 0.1. All seawater

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155 was pumped from the Yaquina Bay, Newport, passed through 10 µm bag filters, and aerated
156 with soda lime air overnight to adjust the pH. In the experiments described in 2.4. and 2.5., the
157 larvae were hatched in autoclaved seawater with 2 µg/mL chloramphenicol and 10 µg/mL
158 ampicillin. For the experiment described in 2.7, gametes were collected aseptically using
159 ethanol for gonad surface-disinfection and sterile instruments (Douillet & Langdon, 1993), and
160 the incubation seawater was autoclaved.

161 After incubation, one-day-old (24 hours post-fertilization; hpf) D-larvae were collected on a 45
162 µm sieve and thoroughly rinsed with sterile seawater. Larvae from different parental crosses
163 were pooled in equal proportions and then distributed among culture containers at a density of
164 5 to 35 larvae per mL. The culture containers varied from 1 mL to 30 L depending on the type of
165 assay. Twenty-four hpf D-larvae were used in all the infection assays.
166

167 2.4. Screening putative probiotic candidates for potential 168 pathogenicity against *C. gigas* (Miyagi stock) larvae using well-plate 169 assays

170 Probiotics were tested for pathogenicity as described by Estes et al. (Estes et al., 2004), with
171 minor modifications. A suspension of 24 hpf D-larvae was diluted to 35 larvae per mL with 10
172 µm-filtered and autoclaved seawater, and one mL of the larval suspension was added to each
173 well of 24-well plates (Corning, USA). Candidate probiotics were each added at a concentration
174 of 3 x 10^4 CFU/mL. Each treatment was replicated six times. After 48 hours of incubation at 25
175 °C, larvae were preserved in their wells with 0.1% (v/v) buffered formalin (pH 8.2). Live and

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176 dead larvae were counted by light microscopy (Leica DMIL LED inverted microscope, x10
177 objective). Tissues of dead larvae degraded rapidly, facilitating differentiation between live and
178 dead larvae. If greater than 90% of tissue remained within the shells, the larvae were classified
179 as having been alive. If less than 90% of tissue remained, the larvae were classified as having
180 been dead before formalin-preservation (Madison et al. 2022).
181
182 Each assay in this study contained a larvae-only negative control, which did not receive any
183 bacteria and was used to normalize larval mortalities that were unrelated to experimental
184 treatments. This control enabled calculation of relative percent survival (RPS). RPS was
185 calculated as RPS = [1- (percent mortality of treatment group / percent mortality of untreated
186 control group)] x 100. In addition, positive controls were included with larvae that were
187 exposed to *V. coralliilyticus* without addition of probiotics.
188

189 2.5. Screening putative probiotic candidates for protection of *C. gigas* 190 (Miyagi stock) larvae against *V. coralliilyticus* strain RE22 using well- 191 plate assays

192 Protective activities of the probiotic candidates were determined in well-plate assays by adding
193 the probiotic candidates (3 x 10^4 CFU/mL final probiotic concentration) to 24 hpf D-larvae in
194 sterile seawater, followed at 48 hpf by addition of *V. coralliilyticus* strain RE22 at a
195 concentration of 6 x 10^3 CFU/mL. The larvae were then incubated at 25 °C for 48 hours, after
196 which time they were preserved with 0.1% buffered formalin. Live and dead preserved larvae

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197 were observed and counted by light microscopy (Leica DMIL LED inverted microscope, x10
198 objective).
199

200 2.6. Identification of probiotic candidates by 16S rRNA gene 201 sequencing

202 Genetic identification of the candidate probiotics D16, DM14, B1, and B11 was conducted by
203 16S RNA gene sequencing and the NCBI's BLAST suite (Altschul et al., 1990). DNA was extracted
204 using phenol:chloroform extraction from one mL of overnight culture according to published
205 protocols with the slight modification that after the final thaw during the RNAse step, 20 µg/mL
206 RNAse A was added, and samples were incubated at 34°C for 30 min (Crump et al., 2003).
207 Amplification of the 16S rRNA gene from each genome was performed under standard PCR
208 conditions with the forward primer 8F 5'-AGAGTTTGATCCTGGCTCAG and the reverse primer
209 1513R 5'-ACGGCTACCTTGTTACGACTT amplifying an approximately 1500 bp piece of DNA.
210 Dideoxy sequence reads were generated from the cleaned PCR product using the same primers.
211 The forward and reverse sequence reads were assembled and trimmed, and the resulting
212 consensus sequence was then queried against the NCBI's 16S ribosomal RNA sequence
213 database using BLASTN (Altschul, 1990).
214

215 2.7. Developing a probiotic combination treatment using well-plate 216 assays

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217 Promising candidates were tested in combinations to potentially take advantage of combining
218 different probiotic modes of action. Each probiotic test well was filled with a one mL suspension
219 of approximately 35 one-day-old (24 hpf) D-larvae and probiotic isolates (B11, DM14, and D16)
220 in sterile seawater. These probiotics were tested individually or combined in equal
221 concentrations of two or three probiotics to achieve final total probiotic concentrations of 3 x
222 10^4 CFU/mL. At 48 hpf, the probiotic-treated larvae were challenged with *V. coralliilyticus* strain
223 RE22 and incubated until 96 hpf. A positive control consisted of larvae with additions of *V.*
224 *coralliilyticus* alone (Vcor only). Lastly, a negative control was included that consisted of larvae
225 that did not receive probiotics nor *V. coralliilyticus* (Larvae only). At 96 hpf, larvae were
226 preserved with the addition of buffered formalin and live and dead larvae were counted, as
227 described above.
228

229 2.8. Effects of the probiotic combination on growth, settlement and 230 metamorphosis of *C. gigas* (Miyagi stock) larvae in a long-term assay

231 2.8.1. Larval culture and experimental treatments

232 Larvae (24 hpf) were stocked at a concentration of five larvae per mL in 10-L containers filled
233 with 10 µm-filtered seawater, with five replicates per treatment group. One treatment group
234 received probiotics added to the larval culture water once at 24 hpf (Single PB Addition). The
235 other treatment group received probiotics following water changes every 48 hours (Multiple PB
236 Additions). A control treatment of larvae alone (Larvae Only), without additions of probiotics,
237 was also included.

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238

239 Probiotics were prepared as previously described but were applied to the 10-L cultures at a
240 concentration of 6 x 10^4 CFU/mL each, resulting in a total combination treatment of 1.8 x 10^5
241 CFU/mL. The first water change occurred 72 hours after stocking the larvae, then every 48
242 hours after that. During water changes throughout this experiment, larvae were poured over a
243 45 µm screen to avoid removing slow-growing larvae. Larvae were rinsed with 10 µm-filtered
244 seawater at each water change. As part of the water change, the containers were scrubbed
245 with 0.02% (v/v) Vortexx (Ecolab, USA) and thoroughly rinsed with hot tap water. In addition,
246 the airlines were rinsed with tap water before the containers were refilled with fresh seawater.
247 Metamorphosis success was determined on 20, 22 and 24 dpf.

248

249 Larvae were cultured according to established methods (Langdon et al., 2003). Briefly, larvae
250 were fed during the first six days on an algal diet of the flagellate *Tisochrysis lutea* (strain C-ISO)
251 at 4 x 10^4 cells/mL. On day seven, the diet was modified to include a 50/50 mixture (by cell
252 concentration) of C-ISO and the diatom *Chaetoceros gracile* and the total cell concentration was
253 increased to 50,000 cells/mL and then further increased to 80,000 cells/mL on day twelve.

254

255 **2.8.2. Larval growth**

256 Random larval sub-samples were collected from the 10-L containers on day 8 and transferred to
257 24-well plates in an attempt to carry out a challenge assay with larvae exposed to *V.*
258 *coralliilyticus* over a four-day period. This assay failed because no mortalities were observed in
259 any treatments or the positive control (Vcor only) (data not presented). Control larvae from

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260 wells that were not exposed to *V. coralliilyticus* RE22 were sampled on day 12, preserved with
261 0.1% phosphate-buffered formalin (pH 8.2), and photographed at 40X objective magnification
262 (Leica DMIL LED inverted microscope; Leica DFC400 camera; Leica Application Suite 4.8 Leica,
263 Germany). Over 350 larvae were measured from each treatment. Shell lengths (defined as the
264 greatest dimension parallel to the shell hinge) of larvae were measured using the software
265 Image Pro Premier 9 (Media Cybernetics, USA).
266

267 2.8.3. Larval settlement and metamorphosis

268 Sixteen days post-fertilization (dpf), 4 x 4-inch marble tiles were added to each 10-L container,
269 and larvae larger than 240 µm began setting naturally. On 20 dpf, the marble tiles were
270 removed and photographed. The settled larvae on each tile were counted using the imaging
271 analysis software Image Pro Premier 9 (Media Cybernetics, USA). In addition, larvae set on the
272 buckets and airlines were counted manually during each water change. Spat that set on the
273 buckets, beakers, and airlines as well as spat that settled on tiles were categorized as naturally
274 set.
275

276 After removing the tiles containing the spontaneously settled larvae, 2 x 10^{-4} M epinephrine
277 (Epi) was used to induce the metamorphosis of remaining larvae on days 20, 22, and 24 (Coon
278 et al., 1986). Non-metamorphosing larvae were returned to their culture containers on days 20
279 and 22. Any larvae that had not metamorphosed after exposure to epinephrine on day 24 were
280 counted as larvae. Larvae that metamorphosed were moved to a flow-through upweller
281 system, where they were cultured until 30 dpf to allow production of additional shell growth

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282 These spat were categorized as successfully metamorphosed with epinephrine, preserved in
283 0.1% phosphate-buffered formalin (pH 8.2), and counted.
284

285 2.9. Effects of the probiotic combination on larval settlement and 286 metamorphosis of the Miyagi and Midori stocks of the Pacific oyster 287 (*Crassostrea gigas*) and the Kumamoto oyster (*Crassostrea sikamea*) 288 in long-term assays

289 The experiment described above (section 2.8) was repeated with larvae of two different stocks
290 of the Pacific oyster - the commonly farmed Miyagi stock and the newly introduced Midori
291 stock (de Melo et al., 2021), as well as larvae of the Kumamoto oyster. The experimental
292 conditions were similar to those described above; however, there were several differences: 1)
293 the larvae were raised in 30 L of seawater, and the first water change occurred 48 hours after
294 stocking the larvae, 2) live *Nannochloropsis occulata* (Nanno) cells were added to the C-ISO diet
295 on days two to six, 3) the algal diet was checked daily and, if the larvae had consumed all food,
296 the total cell concentration of the algal diet was increased by 5000 cells/mL from an initial
297 concentration of 35000 cells/mL C-ISO, and 15000 cells/mL Nanno and, 4) larval densities were
298 intentionally reduced during the experiment to be more similar to culture practices of
299 commercial oyster hatcheries where slow-growing larvae are routinely discarded by sieving
300 (Barton et al. 2012).
301

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302 Larvae were initially stocked at five larvae per ml of seawater, as in the long-term Pacific oyster
303 assay described above. Slow-growing larval *C. gigas* were removed from the cultures if they
304 were not retained on a 64 µm-screen on day 7 or on an 80 µm-screen on day 9. Larval *C.*
305 *sikamea* are smaller than larval *C. gigas*; therefore, larvae that were not retained a 64 µm-
306 screen were removed later on day 9. After removing all slow-growing larvae, the remaining
307 larvae were reduced to a density of one larva per ml on day 9. Slow-growing larvae that were
308 subsequently not retained on a 180 µm-screen on day 17 were removed. Larvae grew faster in
309 this experiment, so the tiles were added on day 15 and removed on day 17 post-fertilization.
310 Subsequently, three rounds of epinephrine were used to induce metamorphosis on days 17, 19,
311 and 21 post-fertilization.
312

313 2.10. Statistical analyses

314 Data from larval survival assays was converted to relative percent survival (RPS), using the
315 calculation described previously. Relative percent survival was arcsine-square-root transformed
316 prior to analysis. Statistical analyses were conducted using R statistical software (Version 4.0.3,
317 R Project for Statistical Computing). Graphs were generated using the ggplot2 R package.
318 Normality was assessed using the Shapiro-Wilk test and Q-Q plots. Homogeneity of variance
319 was assessed using Levene's test from the car.R package. When no significant violations of the
320 assumptions of parametric tests were observed, multiple comparisons of treatment group
321 means against control groups were conducted using Dunnett's test, with the nCDunnett R
322 package. When multiple comparisons between all treatment group means were of interest,
323 data was first fitted to a linear model, then a one-way analysis of variance was conducted to

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324 determine if significant differences between treatment groups existed. If significant differences
325 existed, Tukey's honest significant difference test was applied to obtain pairwise comparisons
326 of treatment groups.
327
328 When significant deviations from the assumptions of parametric tests were observed in data
329 sets, nonparametric methods were used for statistical analyses. For non-parametric multiple
330 comparisons of treatment groups, the Kruskal-Wallis one-way analysis of variance was
331 conducted to determine if significant differences between treatment groups existed. Pairwise
332 comparisons between treatment groups were then conducted using Dunn's test with the
333 Benjamini-Hochberg correction applied, from the dunn.test R package.
334
335 For data with no significant violations of normality, comparisons between only two treatment
336 group means were conducted using a two-sample t-test. When heterogeneity of variance
337 between both treatment groups was observed, Welch's two-sample t-test was applied to
338 compare treatment group means. Comparisons between two treatment groups where the data
339 showed significant violations of normality were conducted using the Mann-Whitney U test.
340

341 3. Results

342 3.1. Initial screening of probiotic candidates

343 From all microbial strains collected for probiotic screening, approximately 28.3% were not
344 revivable after freeze-thawing or failed to sufficiently grow in LBSw within 48 hours, 36.7%

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345 grew on TCBS agar and were consequently excluded, leaving 45% screened on agar plates
346 against *V. coralliilyticus* strain RE22. Ultimately 13 strains were suggestive of contact inhibition
347 or zones of clearing on the *V. coralliilyticus* lawn. These strains proceeded to pathogenicity
348 testing with oyster larvae (project workflow in S1 Figure).
349

350 3.2. Screening putative probiotic candidates for potential 351 pathogenicity against *C.gigas* (Miyagi stock) larvae using well-plate 352 assays

353 Putative probiotic candidates DM7, DM36, DM39, and D20 passed the agar inhibition test, but
354 when added to larvae 24 hpf, caused a reduced survival that was statistically lower than that of
355 the non-probiotic control (Larvae Only). As a result, these candidates were excluded from
356 further evaluation (S1 Table).
357

358 3.3. Screening putative probiotic candidates for protection of *C. gigas* 359 (Miyagi stock) larvae against *V. coralliilyticus* strain RE22 using well- 360 plate assays

361 The highly virulent *V. coralliilyticus* strain RE22 was added to two-day-old larvae (48 hpf) in well
362 plate assays, at a concentration of $6x10^3$ CFU/mL 24 hours after probiotic additions at 3×10^4
363 CFU/mL. The positive control (Vcor only) did not receive any probiotics and resulted in almost

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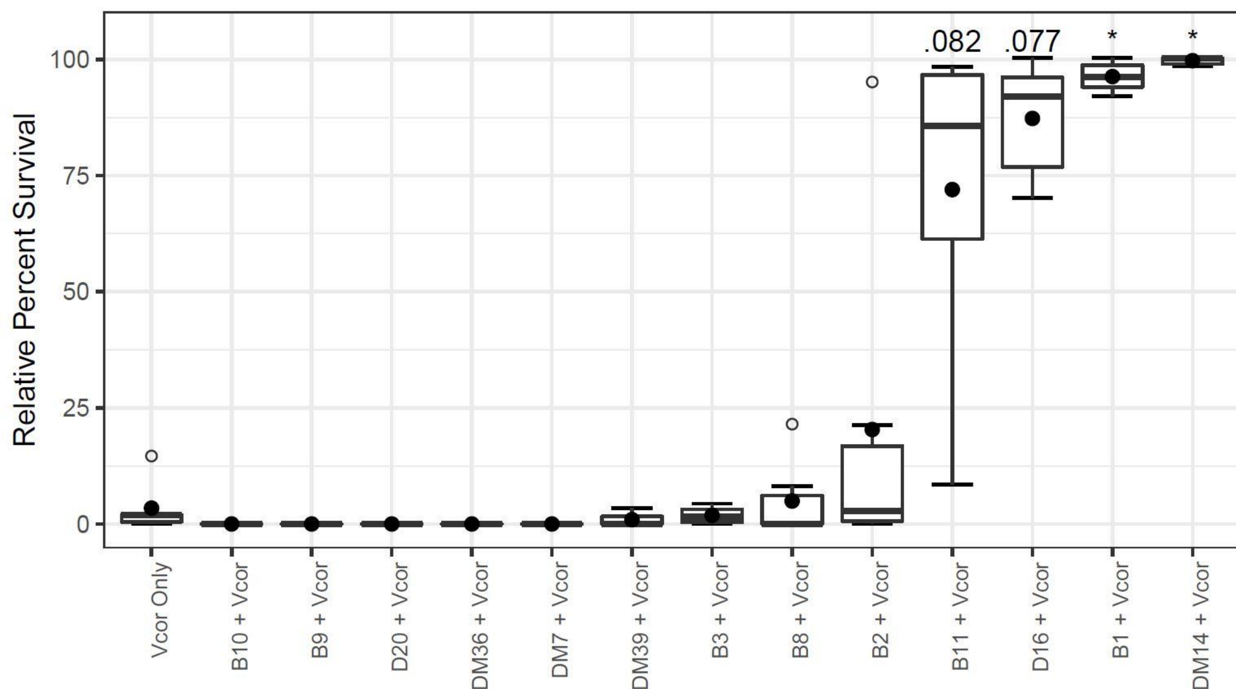
364 complete larval mortality, reducing the relative percent survival to an average of 3.40% (Figure
365 1).



366
367 **Figure 1: *C. gigas* larvae were protected against *V. coralliilyticus* RE22 (Vcor) with probiotic candidates**
368 **B11, D16, B1, and DM14.** Probiotic candidates were evaluated in well-plate assays with 24 hpf-old *C.*
369 *gigas* larvae. "Vcor Only" was the positive control with no additions of probiotics but was inoculated
370 with *V. coralliilyticus* 48 hpf. A negative control did not receive any probiotics or pathogen and was used
371 to normalize mortalities that were not related to pathogen or probiotic treatments to calculate relative
372 percent survival (RPS) values. Filled circles depict the average relative percent survival of six replicate
373 wells. The boxes indicate the upper and lower quartiles, and the bar represents the median or middle
374 quartile. The ends of the whiskers represent the most extreme values within the 1.5x interquartile range
375 (IQR), and the empty circles indicate outliers. Only the treatments using the probiotic candidates B11,
376 D16, B1, and DM14 yielded more than 68% improved average RPS, compared to the positive control
377 (Vcor Only). However, statistical analysis using Dunn's Test with a Benjamini-Hochberg Correction
378 resulted in no (D16 P=.077; B11 P=.082) or low (B1/DM14 .05>P>.01; indicated with asterisks)
379 statistically significant differences between larval survival with the probiotic treatments and the "Vcor
380 Only" control, due to the high variance of some of the treatments (S2 Table).

381

382 Of the 13 remaining candidates tested in this experiment, additions of the four probiotic
383 candidates DM14, B1, D16, and B11 each resulted more than a 68% increase in mean RPS
384 compared to that with the positive *V. coralliilyticus* control (Vcor Only) (S2 Table). Treatments

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385 with DM14 or B1 resulted in minimal larval losses and an average survival of 99.71 ± 0.87% and
386 96.29 ± 3.30%, respectively (S2 Table). In contrast, larvae treated with D16 or B11 showed high
387 variabilities in survival among replicates (S3 Table). Larvae treated with D16 had a slightly
388 reduced survival in two replicate wells (71.8% and 70%), but high survival in the remaining four
389 test wells leading to a mean RPS of 87.28 ± 12.96% (S2 and S3 Tables). Lastly, larvae treated
390 with B11 had high survival in three wells (95.31%, 96.77%, 98.15%), moderate in two wells
391 (56.4% and 75.47%), and a low survival of 8.5% in one well, leading to a mean RPS of 71.96 ±
392 35.10% (S2 and S3 Tables). Repeated trials, including the use of different seawater sources
393 (data not shown) did not reduce inter-replicate variabilities observed for all probiotic
394 candidates. Probiotic candidates were, therefore, advanced to further testing when mean RPS
395 values were at least 40%, regardless of whether a statistical significance of $P<.05$ was achieved.
396 The addition of candidates B2, B3, B8, B9, B10, DM7, DM36, DM39, and D20 did not result in an
397 40% improved mean RPS compared to the positive control (Vcor Only), and these isolates were
398 consequently excluded from further testing (S2 Table).
399

400 3.4. Identification of the probiotic candidates by 16S rRNA gene 401 sequencing

402 After the screening of the putative probiotics against *V. coralliilyticus* in larval assays, promising
403 probiotic candidates were submitted to 16S rRNA sequencing. BLASTN suite search identified
404 D16 and DM14 as different *Pseudoalteromonas* spp., and B11 as an *Epibacterium* sp. Finally,
405 isolate B1 was identified as a *Vibrio* sp. and was consequently excluded at this stage.

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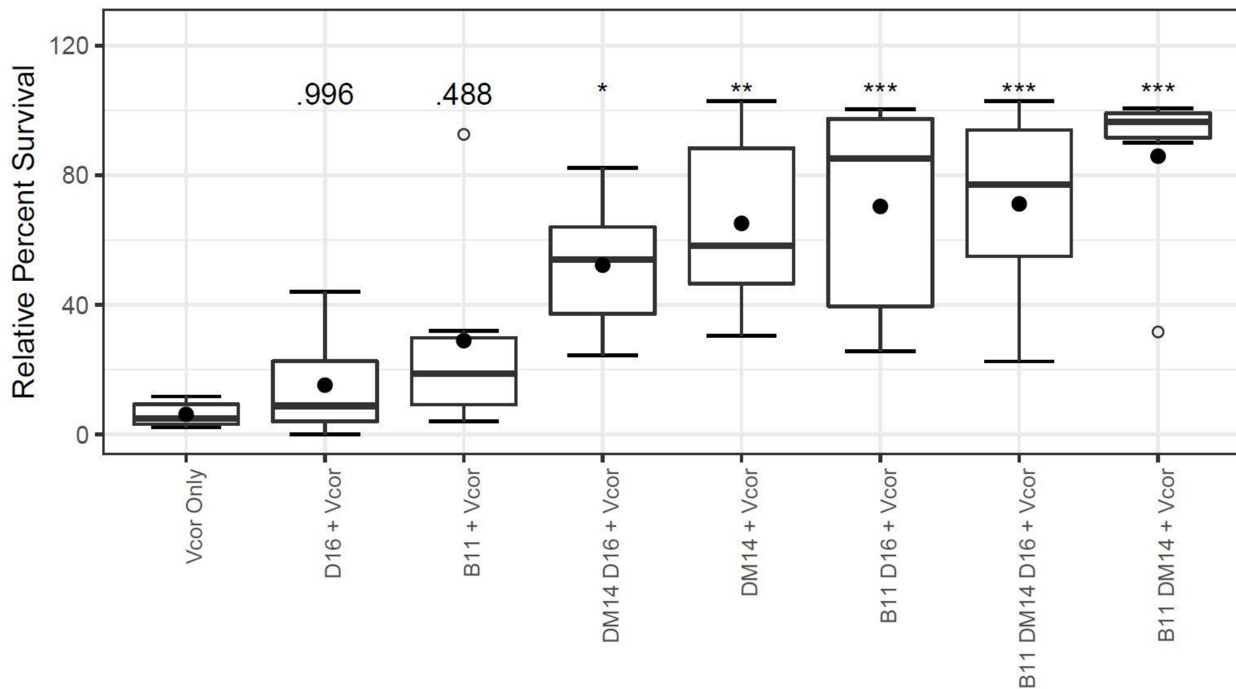
406

407 3.5. Developing a probiotic combination treatment using well-plate 408 assays

409 Individual or combination treatments of the candidate probiotics B11, D16, and DM14 were
410 evaluated for their ability to reduce larval mortalities due to *V. coralliilyticus* and to decrease
411 variability among replicates (Figure 2).



412
413 **Figure 2: Relative percent larval survival with individual or combination probiotic treatments against**
414 ***V. coralliilyticus* strain RE22 for 1-day-old D-larvae of *C. gigas*.** Individual or combinations of
415 probiotics were evaluated for their ability to decrease larval mortalities due to *V. coralliilyticus*.
416 "Vcor Only" was the positive control that did not receive any probiotics. Filled circles depict the
417 average relative percent survival of six replicate wells. The boxes indicate the upper and lower
418 quartiles, and the bar represents the median or middle quartile. The ends of the whiskers
419 represent the most extreme values within the 1.5x interquartile range (IQR), and the empty
420 circles indicate outliers. *, ** and *** indicate statistical differences from "Vcor Only" at *P*≤.05,
421 *P*≤.01, and at *P*≤.001, respectively.

422

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423 Oyster larvae, that did not receive any probiotics 24 hpf but were challenged with *V.*
424 *coralliilyticus* 48 hpf, showed a low relative percent survival (RPS) averaging 6.23% across the
425 six replicate wells (S5 Table). Individual applications of D16 and B11 did not increase the mean
426 RPS of the larvae compared to the positive control (Vcor only) (15.24%; *P*=.996) and (28.98%;
427 *P*=.488), respectively. Only the individual probiotic candidate DM14 significantly increased the
428 mean RPS (65.17%; *P*=.003). However, when D16 and B11 were combined the mean RPS was
429 64.17% higher than the positive control (*P*=.001), and combining DM14 and D16 resulted in a
430 46.05% improvement (*P*=.034). Combining B11 and DM14 achieved a mean RPS of 85.88%
431 (*P*<.001); however, one of the replicates was categorized as an outlier (RPS of 31.67%; data not
432 shown). The three-strain combination resulted in a mean RPS of 71.18% (*P*=.001) (S5 Table).
433 Overall when the results of the individual, two-strain, and three-strain combinations were
434 combined (S2 Figure), there was no statistical significance of the single strain additions
435 compared to the positive control (*P*=.085). The effects of the two- and the three-strain
436 combinations were similar to each other (*P*=.999) but significantly different from that of the
437 Vcor only control (S6 Table).

438

439 3.6. Effects of the probiotic combination on growth, settlement and 440 metamorphosis of *C.gigas* (Miyagi stock) larvae in long-term assays

441 3.6.1. Larval growth

442 Larvae from the five replicate control containers that did not receive any probiotic treatment
443 had an average shell size of 153.21 ± 28.52 µm, which was significantly smaller than those of

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444 the treatment groups that received either a single dose or multiple doses of probiotics
445 (Dunnett's Test, $P \leq .001$) (Table 1). The treatment group that received repetitive probiotic doses
446 after each water change had an average shell size of 161.24 ± 30.89 µm, and the treatment
447 group that received a single probiotic addition 24 hpf resulted in a value statistically similar to
448 the repetitive dosing treatment ($P = .258$), but with slightly greater average shell length of
449 164.49 ± 30.81 µm (Table 1) (S3 Figure).
450

451 Table 1: Average shell lengths of *C. gigas* (Miyagi stock) larvae after probiotic treatments.

Dunnett's Test (comparing to Larvae Only control)					
Treatment	**Mean shell Length (µm)**	**Standard Deviation**	**Difference**	**95% Confidence Interval (CI)**	***p*-value**
Single PB Addition	164.49	30.81	11.28	(6.57, 16.00)	**<.001*** **
Multiple PB Additions	161.24	30.89	8.03	(3.15, 12.92)	**.001*** **
Larvae Only	153.21	28.52			
Tukey-Kramer (pairwise comparisons between PB treatments)					
	Pairwise Comparison		**Difference**	**95% CI**	***p*-Value**
	PB Multiple - PB Single		-3.25	(-8.09, 1.60)	.258

452 *** indicates $P \leq .001$
453

454 **3.6.2. Larval settlement and metamorphosis**

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455 Spat were categorized as undergoing successful metamorphosis when they survived until 30
456 dpf, i.e., for at least six days after settlement or addition of epinephrine. An average of 2.24% of
457 larvae in the control treatment group that did not receive any probiotics naturally set on the
458 marble tiles and surfaces of the culture container (Figure 3) (S7 Table). The remaining larvae
459 exposed to epinephrine displayed a successful metamorphosis rate of 4.81%, resulting in a
460 combined metamorphosis rate of 7.06%. A one-time probiotic addition at 24 hpf resulted in
461 8.29% natural settlement and metamorphosis. When epinephrine was added to the remaining
462 larvae, 13.03% metamorphosed, resulting in a combined metamorphosis rate of 21.32% that
463 was significantly higher than that of the control without addition of probiotics (P=.004).
464 Repeated dosing of the probiotic combination after each water change resulted in a successful
465 settlement and metamorphosis rate of 2.8% of larvae on marble tiles. The addition of
466 epinephrine yielded a metamorphosis rate of 4.24%, resulting in a total metamorphosis rate of
467 7.04%, which was not significantly different from the "Larvae Only" control (P>.999) (S7 Table).

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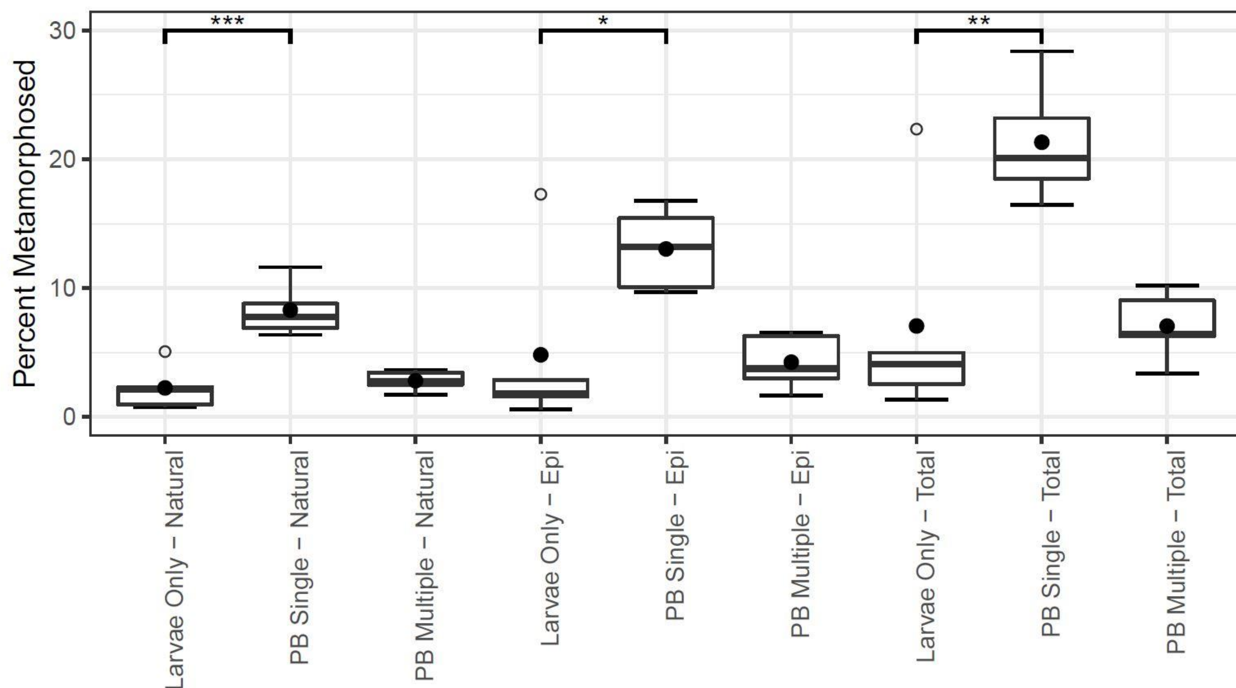

468

469 **Figure 3: Single application of the probiotic (PB Single) improved settlement and**
470 **metamorphosis of *C.gigas* (Miyagi stock) larvae.** A single dose of the probiotic combination (PB
471 Single) 24 hpf significantly improved successful metamorphosis of naturally set (*P*<.001***) and
472 epinephrine-treated (*P*=.028*) *C. gigas* larvae, resulting in significantly improved total
473 metamorphosis (*P*=.004**), compared with the control with no probiotic additions.
474 Metamorphosis success with repeated probiotic applications (PB Multiple) did not differ from
475 that of control larvae (Larvae Only) (*P*>.999) (S7 Table). Filled circles depict the average relative
476 percent survival of five replicate 10-L containers. The boxes indicate the upper and lower
477 quartiles, and the bar represents the median or middle quartile. The ends of the whiskers
478 represent the most extreme values within the 1.5x interquartile range (IQR), and the empty
479 circles indicate outliers.

480

481 3.7. Effects of the probiotic combination on larval settlement and 482 metamorphosis of *C. gigas* (Miyagi and Midori stocks) and *Crassostrea* 483 *sikamea* (Kumamoto) in long-term assays

484 The addition of a single dose of the B11, DM14, D16 probiotic combination 24 hpf increased the
485 percentage of larvae that metamorphosed successfully in Miyagi and Midori stocks of *C. gigas*

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486 as well as the Kumamoto oyster ($P \leq .02$) (Table 2). Because the larval density was reduced to
487 one larva per mL on day nine post-fertilization, the percent metamorphosed was based on the
488 number of larvae restocked on that day.
489

490 Table 2: Settlement and metamorphosis of larvae from two stocks of *C. gigas* (Miyagi and
491 Midori) and the Kumamoto oyster (*C. sikamea*) were improved with a single addition of
492 probiotics at 24 hpf.

Oyster species/stocks	Treatment	Natural Set Avg. % (±%STDEV)	Metamorphosis with Epi Avg. % (±%STDEV)	Total Metamorphosed Avg. % (±%STDEV)	Two-sample t-test *p*-value[b]
Miyagi[a]	w/ probiotics	24.02 (±11.18)	55.74 (±10.51)	79.76 (±6.03)	.020
Miyagi[a]	no probiotics	20.89 (±6.18)	43.21 (±8.38)	64.10 (±10.41)	
Midori	w/ probiotics	22.21 (±11.82)	34.46 (±6.54)	56.67 (±6.37)	.015
Midori	no probiotics	15.34 (±2.44)	29.36 (±7.42)	44.70 (±5.81)	
Kumamoto	w/ probiotics	26.18 (±3.79)	31.72 (±4.99)	57.90 (±4.04)	.004
Kumamoto	no probiotics	21.31 (±2.82)	24.82 (±4.20)	46.14 (±5.03)	

493 [a]Miyagi is the stock name of the commonly cultured Pacific oyster (*C. gigas*) on the US West
494 coast, while the Midori stock was introduced to the US West coast in the early 2000s (de Melo
495 et al., 2021); [b]Two-sample t-tests were conducted on the total ratios of set larvae. All other t-
496 tests in S8 Table.
497

498 3.7.1. Effects on *C. gigas* Miyagi stocks

499 Larvae in the control treatment group that did not receive any probiotics showed a rate of
500 20.89% natural settlement and metamorphosis on marble tiles (S4 Figure). Non-settled larvae

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501 were then treated with epinephrine and 43.21% underwent successful metamorphosis. The
502 subsequent total metamorphosis of larvae without any probiotics was 64.1%. A one-time
503 probiotic addition 24 hpf resulted in 24.02% natural settlement. When epinephrine was added
504 to the remaining larvae, 55.74% metamorphosed, resulting in a combined metamorphosis rate
505 of 79.76% that was significantly higher than total metamorphosis without addition of probiotics
506 (*P*=.020) (Table 2).

507

508 **3.7.2. Effects on *C. gigas* Midori stocks**

509 The probiotic treatment resulted in an average settlement and metamorphosis rate of 22.21%
510 for naturally set Midori larvae versus 15.34% for non-probiotic treated controls (S5 Figure).
511 With epinephrine, 34.46% of the larvae successfully metamorphosed compared to 29.36%
512 without a probiotic addition. The total metamorphosis of Midori larvae treated with probiotics
513 was 56.67% versus 44.7% (*P*=.015) (Table 2).

514

515 **3.7.3. Effects on *C. sikamea* (Kumamoto) oysters**

516 With Kumamoto oysters, the probiotic treatment increased the proportion of successfully
517 naturally set and metamorphosed larvae to 26.18% compared to 21.31% of non-probiotic
518 treated larvae (S6 Figure). The epinephrine treatment resulted in 31.72% metamorphosis
519 versus 24.82% for non-probiotic treated larvae. The total proportion of successfully
520 metamorphosed spat was 57.9% with the addition of probiotics, while non-probiotic treated

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521 Kumamoto larvae had a significantly lower total metamorphosis success of 46.14% (*P*=.004)
522 (Table 2).
523

524 4. Discussion

525 This study describes the development of a promising novel probiotic treatment for both disease
526 prevention and enhancement of larval development. The treatment consisted of a combination
527 of three individual beneficial bacterial isolates added to the larvae culture once at 24 hpf. This
528 treatment significantly improved the survival of Pacific oyster larvae exposed to a lethal dose of
529 a highly virulent *V. coralliilyticus* strain RE22 added 24 hours after the probiotic addition. In
530 addition, a single dose of the probiotics 24 hpf improved subsequent growth of Pacific oyster
531 larvae and resulted in higher larval settlement and metamorphosis success for two stocks of
532 Pacific oysters and for the Kumamoto oyster. Larval metamorphosis and post-settlement
533 survival are common seed production problems for commercial hatcheries (Barton et al. 2012).
534 Initial selective steps in choosing probiotic candidates in this study included survival of freeze-
535 thaw steps, absence of pathogenicity towards larvae, and the ability to suppress the growth of
536 *V. coralliilyticus* strain RE22 using agar plate assays. Due to the various possible modes of
537 probiotic action, agar plate assays are arguably not definitive in predicting pathogen
538 suppression in culture systems because other modes of action could be overlooked in the
539 process. In addition, growth on the *Vibrio*-selective agar (TCBS) led to the exclusion of
540 candidates with antagonistic properties. Due to likely intense interspecies competition in this
541 genus (see, for example, Borgeaud et al. 2015), *Vibrio* spp. often displayed moderate to strong

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542 inhibition of the target strain on agar plates and *Vibrio* isolate B1 showed promise in survival
543 assays with oyster larvae. For unknown reasons, B1 failed to grow on TCBS plates at the
544 screening stage and was only subsequently identified as a *Vibrio* sp. by sequencing leading to its
545 exclusion from further testing. Members of the genus *Vibrio* were intentionally excluded from
546 the probiotic combination as they have the ability to quickly adapt to new environments and
547 acquire virulence factors by horizontal gene transfer that could potentially transform the isolate
548 from an oyster commensal to a pathogen (Bruto et al., 2017; Le Roux & Blokesch, 2018).

549 Individual probiotic candidates that improved mean RPS in initial screenings by at least 40%
550 compared to a non-probiotic treated control, were selected for inclusion in the probiotic
551 combination. Ultimately, a combination of the three isolates B11, DM14, and D16 significantly
552 increased the efficacy of the treatment. High variations were observed in these assays
553 irrespective of probiotic isolate, larval rearing method, including larval density and rearing
554 volume (well-plates or 10-L containers), or seawater quality (autoclaved or 10 µm-filtered).
555 Such variation seems common in trials with oyster probiotics (Sohn et al., 2016) and may be
556 due to small, uncontrolled differences in environmental conditions among replicates that affect
557 the bacterial composition of the cultures, microbiome or host-microbe interactions (Stevick et
558 al., 2019).

559 Application of probiotics in aquaculture systems, including probiotics tested in oyster
560 hatcheries, are commonly dependent on repeated probiotic dosing (Kapareiko et al., 2011;
561 Sohn et al., 2016). It has been suggested that repeated additions are necessary due to the
562 inability of the probiotic bacteria to permanently colonize the animal's mucosal surfaces,
563 including the gut (Gatesoupe, 1999). This study evaluated whether repeated dosing after each

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564 water change was required for prolonged beneficial effects of the probiotic combination. We
565 found significant improvements in growth at day 12 as well as subsequent settlement and
566 metamorphosis success for *C. gigas* larvae with a single dose of the probiotic combination at 24
567 hpf; however, repetitive dosing was less beneficial. Significantly improved metamorphosis and
568 settlement with a single dose of probiotics at 24 hpf was consistent for larvae from two stocks
569 of *C. gigas* (Midori and Myagi) and *C. sikamea* (Kumamoto) oysters. It is known that bacteria
570 affect bivalve settlement via biofilm formation or secretion of chemical settlement cues (W. K.
571 Fitt et al., 1990; Weiner et al., 1985). However, after multiple water changes, it is unlikely that
572 the initial probiotic dosing between 24 and 72 hpf influenced subsequent biofilm formation or
573 settlement cues more than two weeks later.

574 In the past decade, research efforts have focused on identifying new probiotics intended to
575 increase the survival of oyster larvae exposed to pathogens (e.g., (Karim et al., 2013; Kesarcodi-
576 Watson et al., 2012; Lim et al., 2011)). It has been found that the early establishment of the
577 microbiome in oyster larvae is beneficial to early oyster development and survival (Gomez-Gil
578 et al., 2000; Harris, 1993; Kesarcodi-Watson et al., 2012; Prado et al., 2010; Schulze et al.,
579 2006). Knowledge of the beneficial modes of action of probiotics is sparse, particularly in the
580 aquatic environment when probiotic benefits could be related to interactions with the rearing
581 environment, the host-associated microbial community, or with the host's eukaryotic cells
582 (Gomez-Gil et al., 2000; Lebeer et al., 2010; Prado et al., 2010; Van Doan et al., 2020). These
583 modes could include modulation of the resident microbiome to a more beneficial state, direct
584 antagonism against individual cells of a pathogen, such as killing by direct contact and toxin
585 secretion, production of antimicrobials, or by changing environmental conditions in ways that

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586 limit pathogen growth via competitive exclusion. Probiotics may also directly interact with the
587 eukaryotic cells of the host and contribute to digestion by supplying enzymes, acting as a
588 nutritional source, restoring mucosal integrity and barrier functions, or modulating the immune
589 response (Kesarcodi-Watson et al., 2008; Verschuere et al., 2000).

590 It is conceivable that multiple modes of action are present in an individual probiotic or that a
591 combination treatment, as studied in this work, could take advantage of different modes of
592 action. Further advantages of combination treatments could be due to a broader spectrum of
593 activity against different pathogen strains or species, retention of beneficial activity even if a
594 single probiotic constituent fails, and potentially reducing selective pressures against probiotic
595 isolates that decrease benefits.

596 Further investigations are needed to explore the modes of action of new probiotic treatments
597 in oyster larvae, such as the effect of probiotic additions on the host's microbiome and the
598 microbial communities of the rearing environment (e.g., Modak & Gomez-Chiarri, 2020; Sohn et
599 al., 2016; Stevick et al., 2019). Such research efforts will help support a sustainable oyster
600 industry facing many challenges due to global warming and ocean acidification.

601

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608 Kennedy grant [NA18NMF4270220] awarded to CL.

609

610 Appendix A. Supplementary figures S1 – S6.

611 Appendix B. Supplementary tables S1 – S8.

612

613 CRediT author statement

614 **David Madison:** Conceptualization, Methodology, Investigation, Data curation, Writing –
615 Review & Editing.

616 **Carla Schubiger:** Conceptualization, Methodology, Investigation, Resources, Writing – Original
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619 **Ryan S. Mueller:** Conceptualization, Methodology, Investigation, Resources, Writing – Review &
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622 Supervision, Project Administration, Funding Acquisition.

623

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