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Pathogenic threats and probiotic use in larviculture of the scallop, *Pecten maximus*

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Abstract:

During a series of experiments, bacteriological elements in scallop larval rearing were investigated: larvae susceptibility to pathogens as a function of their age, and the use of probiotic bacteria during larviculture. Younger larvae (d5 PF) were highly more susceptible to pathogenic-challenge than their older siblings, which were challenged at an older age (d15 PF). A challenge with 10^4 CFU mL⁻¹ of *V. pectenocida* killed 100% of d5 PF larvae 7 days following challenge, yet killed only 9% of d15 PF larvae 9 days following challenge. Use of the probiotics *Phaeobacter gallaeciensis*, *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, provided for larger larvae, a high yield of competent larvae and, perhaps more importantly, protection against pathogen-challenge similar to levels achieved from antibiotic use. When challenged with *V. pectenocida*, d29 survivals were 20.3%, 85.1% and 75.0% respectively for control (no probiotic), antibiotic treated, and 'probiotic mix' administered larvae. Use of potential probiotic *Pseudoalteromonas* sp. D41 appeared to hinder scallop larvae. Future use of probiotics in scallop larval rearing would benefit from combined use of *P. gallaeciensis*, *A. macleodii* 0444 and *Neptunomonas* sp. 0536.

Keywords: scallop ; *Pecten maximus* ; Probiotics ; *Alteromonas macleodii* ; *Neptunomonas* ; *Phaeobacter*

1. Introduction

The worldwide market for scallops represents 2.4 million tonnes p.a. of which a very large proportion (66%) derives from aquaculture (FAO Fisheries & Aquaculture Information & Statistics Service 2010). This large contribution by aquaculture mainly involves on-growing scallops from the wild, as opposed to the dredging of sea beds, which is how the capture fisheries operate. The main countries for scallop aquaculture are China (82% of total aquaculture) and Japan (16%); while Chile, Peru, Canada, Sth. Korea, Russia, Brazil, Ireland, United Kingdom, Norway and Spain also have established industries. With these statistics in mind, it can be seen that there is benefit to be had in many countries from the hatchery production of scallop larvae. Such production would greatly assist the aquaculture-lopsided industry through means

71 of a reliable product (avoiding seasonal fluctuations/spat collection inconsistencies)
72 and stock enhancement through selective breeding.
73
74 Larval production of the great scallop, *Pecten maximus*, does occur at present
75 (Torkildsen & Magnesen, 2004; Merino, Uribe, Soria & von Brand, 2009; Andersen,
76 Christophersen & Magnesen, 2013) but much improvement is still needed.
77 Inconsistent yields plague commercial production with many potential implicating
78 factors; feed quality, broodstock conditioning, water quality/consistency (Andersen,
79 Christophersen & Magnesen, 2011). Current commercial production mostly employs
80 static batch culture rather than continuous flow and the use of antibiotics remains
81 often essential for successful larviculture. However, like other forms of aquaculture,
82 the use of antibiotics needs to be reviewed and alternatives sought (Kesarcodi-Watson,
83 Kaspar, Lategan & Gibson, 2008). Use of probiotic bacteria has been demonstrated to
84 be effective in bivalve larviculture (Riquelme, Jorquera, Rojas, Avendaño & Reyes,
85 2001; Kesarcodi-Watson, Kaspar, Lategan & Gibson, 2010). Recently, it was shown
86 that four species of probiotics were able to protect *P. maximus* larvae against
87 pathogen-challenge by *Vibrio* spp., under the conditions of a multi-well plate bioassay
88 (Kesarcodi-Watson, Miner, Nicolas & Robert, 2012).

89
90 When compared with the larvae of other bivalves such as oysters and mussels, *P.*
91 *maximus* larvae have proven more difficult to culture successfully (Robert & Gérard,
92 1999; Helm, Bourne & Lovatelli, 2004); they appear to be more sensitive to biotic
93 (bacteria) and abiotic factors (tank design/culture technique). The highly variable
94 production output which occurs at present offers risk to the farmer and needs to be
95 improved. To do this, all aspects of scallop larval rearing warrant investigation to find
96 possible areas that allow less variable and improved output. In the present study,
97 bacteriological aspects concerning scallop larval rearing were investigated. Firstly, the
98 effect of the age of scallop larvae upon pathogen susceptibility was investigated.
99 Secondly, the effect of four probiotics was determined during the full larval cycle of
100 scallop. The probiotics were tested both individually and in a multi-strain mix
101 containing all. Each probiotic treatment was tested during routine unchallenged
102 rearing and also against a pathogen-challenge by *V. pectenica*.

103

104 2. Materials and methods

105

106 2.1. *Animals*

107

108 Scallop larvae were obtained from the private hatchery of “Le Tinduff” (Brittany,
109 France) at d2 post-fertilization (PF) when fertilized eggs had hatched to provide D-
110 larvae. Larvae were transported to IFREMER Brest (Brittany, France), for
111 experiments. Between d2 PF and d6 PF, larvae were reared collectively in a 150 l
112 conical tank with gentle aeration (3 l h⁻¹) at a concentration of approximately 13
113 larvae ml⁻¹. Water temperature was maintained at 19 °C, filtered to 1 µm, and U.V.
114 sterilized. Water was changed every two days. At each water change, larvae were fed
115 a 1:1:1 mix of *Pavlova lutheri* (P), *Tisochrysis lutea* (formerly *Isochrysis* aff.
116 *galbana*: T-Iso) (T) and *Skeletonema marinoi* (S), each at 20 cells µl⁻¹.

117

118 Larvae were treated with antibiotics (chloramphenicol 8ppm); one treatment for the
119 “age as a factor of pathogen susceptibility” study at d2 PF, and two treatments for the
120 probiotic trial, at d2 and d4 PF. The treatments were timed to allow three days to pass
121 before experimental pathogen-challenge. Although now banned for commercial
122 application, chloramphenicol was chosen for the present study as it has been used
123 historically on an experimental basis in *P. maximus* larviculture with greater success
124 than other antibiotics (Le Pennec, Prieur & Chardi, 1973; Robert, Miner & Nicolas,
125 1996). Antibiotic treatment, at least once, at the beginning of *P. maximus* larval
126 rearing is currently imperative. Trials rearing scallop larvae without this initial
127 treatment were not successful, even when probiotics were used (unpublished data);
128 this is believed to be a result of the *Vibrio* spp., which can be eliminated by this initial
129 antibiotic treatment.

130

131 2.2. *Bacteriology*

132

133 For trials with probiotic bacteria, four strains of potential probiotics were used:
134 *Alteromonas macleodii* 0444, *Neptunomonas* sp. 0536 (Kesarcodi-Watson et al.,
135 2010), *Phaeobacter gallaeciensis* (Ruiz-Ponte, Cilia, Lambert & Nicolas, 1998),
136 *Pseudoalteromonas* sp. D41 (Kesarcodi-Watson et al., 2012). For trials involving a
137 pathogen-challenge, *V. pectenecida* or *V. splendidus* were used. Strains were revived
138 from -80 °C stores in Marine Broth (Difco) at appropriate temperatures: *A. macleodii*

139 0444, *Pseudoalteromonas* sp. D41 at 37 °C; *Neptunomonas* sp. 0536 at 30 °C; *P.*
140 *gallaeciensis* at 25 °C; *V. pectenica*, *V. splendidus* at 20 °C. Each strain was then
141 sub-cultured three times on Marine Agar (Difco) to ensure purity before use in trials.
142 Prior to use in experiments, bacteria were cultured in 10 ml Marine Broth (Difco) for
143 24 h at the appropriate temperatures. After culture, cells were centrifuged (2500g, 8
144 min, 20 °C) and washed twice with sterile seawater, ready for use in experiments.
145 Washed cells were enumerated following serial dilution and plating onto Marine Agar,
146 allowing the concentrations used for each experiment to be verified.

147

148 2.3. Age as a factor in the pathogen susceptibility of scallop larvae

149

150 It was investigated whether the age of larvae influenced their susceptibility to
151 pathogen-challenge. Larvae originating from the same cohort of *P. maximus* were
152 challenged at two different ages; d5 PF and d15 PF. During the first pathogen-
153 challenge, the larvae were challenged with two pathogens separately, *V. pectenica*
154 and *V. splendidus*, at three concentrations: 10^4 , 10^5 and 10^6 colony forming units ml⁻¹
155 (CFU ml⁻¹). The pathogens were added 24 h after water change and 24 h before the
156 next change. Larval survival was measured for nine days following pathogen-
157 challenge and then the larvae were discarded. Those larvae used in the d15 PF trial
158 originated from the same cohort but had no previous exposure to the pathogen and
159 had been reared in a 150 l conical tank with gentle aeration and water changes every
160 two days. The second pathogen-challenge repeated the challenge with just *V.*
161 *pectenica* (because *V. splendidus* had minimal effect upon d5 PF larvae). The
162 second challenge also employed three concentrations of *V. pectenica* (10^4 , 10^5 , 10^6
163 CFU ml⁻¹). For both trials, flat-bottom beakers (2 l) filled to 1.5 l with 1 µm filtered,
164 U.V. sterilized seawater (19 °C) were used. Larvae were placed at a concentration of
165 10 larvae ml⁻¹. Water changes of all tanks occurred every two days and larvae were
166 fed at water changes as described previously. A control treatment contained non-
167 challenged larvae. All treatments were conducted in triplicate.

168

169 2.4. Probiotic trials during scallop larval rearing

170

171 2.4.1. Probiotic trials

172

173 Larvae were obtained as described in section 2.1. At d6 PF, larvae were placed into 2 l
174 flat-bottom beakers filled with 1.5 l of 1 µm filtered, U.V. sterilized seawater (19 °C).
175 Larvae were placed into each beaker at 10 larvae ml⁻¹. Water was changed every two
176 or three days depending on practicality. Probiotic addition was provided after each
177 water change; first commencing on d6 PF and ceasing on d24 PF. Treatments
178 comprised the probiotics *A. macleodii* 0444, *Neptunomonas* sp. 0536, *P. gallaeciensis*
179 and *Pseudoalteromonas* sp. D41, given individually or in a mix (giving ¼ of the
180 quantity used by each inoculum in the individual treatments into one treatment).
181 Another treatment provided antibiotic administration (chloramphenicol 8ppm) at each
182 water change, without probiotics, allowing comparison to be made between probiotic
183 and antibiotic efficacy. A control treatment contained larvae without addition of
184 probiotic or antibiotic at water changes. All experimental conditions were carried out
185 in triplicate.

186

187 Following preparation for their use in experiments (24 h), each strain was
188 administered at the highest possible concentration (Table 1), determined by the end-
189 concentration of each probiotic culture. Furthermore, because the growth rates of
190 probiotics differed (resulting in a range of end-concentrations), the final “in-tank”
191 concentration of each probiotic ranged between 10⁴-10⁵ CFU ml⁻¹ on the
192 administration days. These concentrations were the same as those tested previously
193 which demonstrated a positive probiotic effect upon scallop larvae (Kesarcodi-Watson
194 et al., 2012).

195

196 Throughout the trial, larval survival was measured via microscope analysis at each
197 water change. Additionally, larval shell sizes were measured on d16, 24, 27 and 29
198 using a Leica DMIL microscope equipped with a CCD camera and image analysis by
199 Image SXM software. The yield of competent larvae, i.e. the proportion of larvae to
200 achieve metamorphosis capability (as determined by appearance of a “double-bar” on
201 the velum in the late stages of larval development), was recorded also at d21, 24, 27
202 and 29.

203

204 TABLE 1.

205

206 2.4.2. Pathogen-challenge trials

207

208 In a concurrent trial, larvae administered probiotics in the same manner as in section
209 2.4.1., were challenged with 10^4 CFU ml⁻¹ *V. pectenicida* on d7 PF (24 h after the first
210 probiotic administration and 24 h before the next water change). Probiotic supply was
211 continued with these larvae until d24 PF. Larval survival and the yield of competent
212 larvae were measured as in section 2.4.1. All treatments were applied in triplicate.

213

214 2.5. Data analysis

215

216 Percent survival figures were arc sin square root transformed to approximate
217 normality. Treatment differences were compared using ANOVA ($p = 0.05$). Post hoc
218 comparisons between survivals were compared using Tukey's test. Larvae size data
219 were compared using nonparametric Kruskal-Wallis analysis. STATISTICA (StatSoft,
220 Inc.), version 7.1 was used for data analysis.

221

222 3. Results

223

224 3.1. Age as a factor of scallop larvae pathogen susceptibility

225

226 Young larvae (d5 PF) from the tested cohort were not highly susceptible to *V.*
227 *splendidus* with only high levels of *V. splendidus* (10^6 CFU ml⁻¹) resulting in a small
228 mortality increase above those in the control treatment (Table 2). However, challenge
229 of d5 PF larvae with *V. pectenicida* resulted in large mortalities at all concentrations
230 (Table 2). Furthermore, at the smallest pathogen inoculum tested (10^4 CFU ml⁻¹) all
231 larvae had died seven days following challenge with *V. pectenicida*. When d15 larvae
232 were challenged, there was a far greater resistance to *V. pectenicida* (Table 2); 10^4
233 CFU ml⁻¹ of the pathogen resulted in just 9% mortality nine days following challenge.
234 Furthermore, pathogen-challenge with a higher level of the bacterium (10^5 CFU ml⁻¹)
235 resulted in only 24% mortality nine days post-challenge in d15 larvae, despite the
236 same level of pathogen obliterating d5 PF larvae just three days following challenge.

237

238 TABLE 2

239

240 3.3. Probiotic trials

241
242 High larval survivals occurred in all treatments during the full larval period (Table 3).
243 There were occasional statistical differences, yet in terms of absolute percentage
244 larval survivals, these differences were very minor and were not present by the end of
245 the trial (d29). Differences were, however, seen between treatments in terms of larval
246 size (Table 3) and the yield of competent larvae (Fig. 1A). The largest larvae were
247 observed in the control and also those administered *A. macleodii* 0444 or *P.*
248 *gallaeciensis* (Table 3). Those provided *Pseudoalteromonas* sp. D41 and the mix of
249 all probiotics grew the slowest. In terms of the competent larvae yields,
250 *Pseudoalteromonas* sp. D41 and the antibiotic treatment performed worse than the
251 other treatments; the other treatments being statistically no different to each other (Fig.
252 1A).

253

254 TABLE 3

255 FIGURE 1

256

257 3.4. Pathogen-challenge trials

258

259 When challenged with *V. pectenicida*, larvae administered chloramphenicol had the
260 highest absolute survivals; however, protection against the challenge was statistically
261 no different to the use of antibiotics when either *P. gallaeciensis* or the probiotic-mix
262 were administered (Table 4). These three treatments provided the best protection
263 against pathogen-challenge; yet, administration of *A. macleodii* 0444, *Neptunomonas*
264 sp. 0536 and *Pseudoalteromonas* sp. D41 also provided protection against the
265 challenge when compared with the untreated pathogen-control larvae (Table 4). In
266 terms of competent larvae yields reached following pathogen-challenge, *P.*
267 *gallaeciensis* and the probiotic-mix performed the best; whereas, the pathogen-control
268 and *Pseudoalteromonas* sp. D41 did the worst (Fig. 1B).

269

270 TABLE 4

271

272 4. Discussion

273

274 To date, attempts at *P. maximus* larviculture have shown the animals to be more
275 sensitive than other commercially reared bivalve mollusks. They have displayed much
276 more vulnerability to bacterial problems, often reliant on antibiotic use (Robert et al.,
277 1996; Merino et al., 2009). The data provided in this study show that factors
278 including: larval age, and the use of probiotics can have a large influence upon the
279 success of a batch of scallop larvae.

280

281 Observation that older larvae had greater resistance to pathogen attack is important.
282 Whilst this occurrence might seem somewhat obvious, we are not aware of other
283 studies which have investigated this aspect. Mollusk larviculture is a vulnerable time
284 for the animals being cultured; however, we can now say, at least with *P. maximus*
285 larvae, that larvae which survive the first two weeks of culture are less likely to
286 succumb to pathogenic bacteria. A likely reason why older larvae withstand pathogen-
287 challenge better than younger larvae is due to the increased lipid levels that are found
288 in older larvae (Marty, Delaunay, Moal & Samain, 1992; Soudant, Marty, Moal,
289 Masski & Samain, 1998). These authors observed significant increases in the total
290 lipids (ng larva⁻¹) of *P. maximus* larvae, reaching as high as a 10-fold increase
291 between d2 and d23 PF (Soudant et al., 1998). As well as nutrition, lipids can have
292 structural, functional and immune properties. Pernet, Bricelj & Parrish (2005) found
293 an increase in arachidonic acid, 20:4(n-6), in larval sea scallop, *Plactopecten*
294 *magellanicus*, and discussed how this PUFA had been implicated in mediation of
295 cellular responses to bacterial infections in insects. Another possible reason for
296 increased pathogen resistance in older larvae might be due to the natural resident
297 bacteria which colonize the larvae. A more developed microflora population might
298 offer natural bacterial defenses against pathogens. Indeed, study of other aquatic
299 animals have shown a shift in dominant bacterial groups during the larval cycle
300 (Bergh, Naas & Harboe, 1994), and perhaps it is the groups which colonize at the later
301 stages which offer enhanced protection against the pathogens. In scallop larvae,
302 bacterial effects have not been studied as highly as lipids; however in the present
303 study we have shown that bacteria have the potential to limit pathogenic effects. It is
304 likely that a natural bacterial population develops in older larvae strengthening them
305 against pathogenic challenges and this is an area which deserves investigation.

306

307 Probiotic use during this study provided advantages to: larval size, yields of
308 competent larvae, and also in protecting larvae against pathogen-challenge. None of
309 the probiotics tested in this study affected the survival of scallop larvae during routine,
310 unchallenged rearing. During routine rearing, the probiotics *P. gallaeciensis* and *A.*
311 *macleodii* 0444 showed merit by producing both the largest larvae and highest yield
312 of competent larvae. Interestingly, despite the larvae inoculated with *Neptunomonas*
313 sp. 0536 not being the largest larvae, they achieved the highest absolute yield of
314 competent larvae. The reason for this is unclear, but it is certainly a useful trait. For
315 these reasons, a probiotic mix is probably a good option for future probiotic use. This
316 mix should exclude *Pseudoalteromonas* sp. D41 which displayed the slowest growth
317 and lowest yield of competent larvae, both being worse than the control larvae. It
318 should be mentioned that a strain of *Pseudoalteromonas* spp. has been described as an
319 opportunistic pathogen of *P. maximus* larvae (Sandaa, Brunvold, Magnesen & Bergh,
320 2008). In the present study, when scallop larvae were challenged with *V. pectenicida*,
321 the probiotic-mix provided high larval survivals which, along with individual
322 administration of *P. gallaeciensis*, protected larvae to a level no different to that
323 observed when antibiotic was used. This benefit was also witnessed in the yields of
324 competent larvae, with the probiotic-mix producing the best performing larvae
325 following pathogen-challenge. It appears that during routine rearing, and also as a
326 protective measure against pathogen attack, administration of a mixture of *A.*
327 *macleodii* 0444, *P. gallaeciensis* and *Neptunomonas* sp. 0536 provides desirable
328 attributes to *P. maximus* larval rearing. Data from the probiotic-mix were potentially
329 undervalued due the mix incorporating *Pseudoalteromonas* sp. D41, which was
330 negative to the larvae, and the benefits provided from a revised probiotic-mix could
331 be investigated in the future.

332

333 Previous studies also investigated probiotics in scallop larviculture (Riquelme,
334 Hayashida, Araya, Uchida, Satomi & Ishida, 1996; Riquelme, Araya, Vergara, Rojas,
335 Guaita & Candia, 1997; Avendaño & Riquelme, 1999; Ruiz-Ponte, Samain, Sánchez
336 & Nicolas, 1999; Riquelme, Araya & Escribano, 2000; Riquelme et al., 2001). All
337 studies except that of Ruiz-Ponte et al. (1999) investigated the Chilean scallop, *A.*
338 *purpuratus*. Like the present study, Riquelme et al. (1996) found a member of the
339 genus *Alteromonas* (*A. haloplanktis*) to be beneficial against pathogen-challenge.
340 Another study by Riquelme et al. (2001) incorporated probiotics into commercial-

341 scale hatchery production using the bacterial strains C33, strain 11, and *Bacillus* sp.
342 (strain B2). The study showed that the probiotics allowed completion of the larval
343 cycle without the need to use antibiotics. Similar to the current study, Ruiz-Ponte et al.
344 (1999) tested *P. gallaeciensis* upon *P. maximus* larvae; however, they found that
345 neither pathogen-challenged nor unchallenged larvae were protected by live bacterial
346 cells of *P. gallaeciensis*. Our findings are in conflict with those of Ruiz-Ponte et al.
347 (1999); herein, *P. gallaeciensis* was the best performing probiotic, providing
348 protection against pathogen attack no different to when antibiotic was used. Ruiz-
349 Ponte et al. proposed that perhaps *P. gallaeciensis* produced substances toxic to the
350 larvae, which were more pronounced at higher levels of *P. gallaeciensis*, or that
351 perhaps the organic matter introduced with higher levels of *P. gallaeciensis* aided in
352 pathogen proliferation. If true, these could potentially explain the differences in their
353 results and those in the current study because they administered *P. gallaeciensis* at 10^6
354 CFU ml⁻¹ whereas 10^4 - 10^5 CFU ml⁻¹ was used in the current study. Additionally, the
355 ambient bacterial community would most certainly have been different between the
356 present study and that of Ruiz-Ponte et al. (1999); and this also might have influenced
357 the effect of probiotic addition.

358
359 One conspicuous aspect of current *P. maximus* larviculture is the use of antibiotics in
360 the early stages. Trials conducted with/without two doses of chloramphenicol (at d3
361 and d4 PF), using the same batch of larvae, showed that those not provided the
362 antibiotic underwent large mortality whilst those provided the antibiotic did not (A.
363 Kesarcodi-Watson, unpublished data). This is a regular occurrence with *P. maximus*
364 larvae (Robert et al., 1996; Torkildsen, Lambert, Nylund, Magnesen & Bergh, 2005)
365 and it appears the *Vibrio* spp. which are present in these early stages (detected by
366 culture on TCBS agar plates) need to be eliminated by antibiotics to allow scallop
367 larvae to proceed successfully. Furthermore, because best efforts are made to sterilize
368 larval rearing waters (1 µm filtered, U.V. sterilized) yet *Vibrio* spp. still occur in early
369 larval rearing waters, it appears that these bacteria enter *P. maximus* larval rearing
370 systems via vertical transmission from the broodstock (Holbach, PhD in prep), also
371 put forward for Chilean scallop, *Argopecten purpuratus* (Riquelme, Hayashida,
372 Toranzo, Vilches & Chavez, 1995).

373

374 Recent work in Norway developed low-exchange continuous-flow techniques for *P.*
375 *maximus* larval rearing which allowed successful larval rearing in the absence of
376 antibiotics (Andersen, Burnell & Bergh, 2000; Torkildsen & Magnesen, 2004;
377 Magnesen et al., 2006; Andersen et al., 2013). During this work, a very low water
378 turn-over (approximately one tank exchange per day) and low density of larvae (3.0-
379 5.2 larvae ml⁻¹ starting density, with a final density at settlement of 1.0 larvae ml⁻¹)
380 were used (Magnesen et al., 2006, Andersen et al., 2013). The work in Norway lays a
381 good foundation to antibiotic-free scallop larviculture (in an industry with increasing
382 restrictions on antibiotic use), but with such low water exchange it currently remains a
383 slightly more intensive version of a batch-culture system; improvements in stocking
384 density and water-flow would be desirable to provide efficient larval rearing. To our
385 knowledge, commercial rearing of scallop larvae by flow-through techniques is
386 currently practiced only in Norway.
387
388 Continued research is needed into scallop larval rearing to facilitate regular and
389 untroubled production. The results from this study provide useful information for
390 direct application in scallop larviculture and also highlight further research avenues.
391

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398

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Table 1. Probiotic concentrations in each treatment throughout scallop larval rearing. Note: d = day, PF = Post-fertilization.

Treatment	"in-tank" probiotic concentration following water changes								
	d6 PF	d8 PF	d10 PF	d12 PF	d14 PF	d16 PF	d19 PF	d21 PF	d24 PF
<i>A. macleodii</i> 0444	2.2×10^5	1.8×10^5	2.6×10^5	1.9×10^5	3.0×10^5	2.9×10^5	1.7×10^5	1.2×10^5	2.7×10^5
<i>Neptunomonas</i> sp. 0536	5.4×10^4	8.6×10^4	3.5×10^4	3.0×10^4	2.5×10^4	2.8×10^4	9.2×10^4	2.0×10^4	6.4×10^4
<i>P. gallaeciensis</i>	1.3×10^5	1.8×10^5	7.0×10^4	7.3×10^4	7.0×10^4	5.0×10^4	6.5×10^4	7.3×10^4	6.0×10^4
<i>Pseudoalteromonas</i> sp. D41	4.5×10^4	1.9×10^4	1.9×10^4	1.7×10^4	1.7×10^4	2.9×10^4	8.4×10^4	2.3×10^5	6.5×10^4
Probiotic mix	¼ of all	¼ of all	¼ of all	¼ of all	¼ of all	¼ of all	¼ of all	¼ of all	¼ of all

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Table 2. Scallop larvae survival (% \pm S.E.) after two separate pathogen-challenges with a range of concentrations. During the first challenge, *V. pectenica* or *V. splendidus* was used. During the second challenge only *V. pectenica* was used. Each challenge used the same cohort of larvae, although different ages of the larvae (i.e. d5 or d15). Values in a column with an asterisk (*) are statistically different to the unchallenged control ($p < 0.05$).

Treatment	First pathogen-challenge (challenged on d5)					Second pathogen-challenge (challenged on d15)			
	1 day post-challenge	3 days post-challenge	5 days post-challenge	7 days post-challenge	9 days post-challenge	1 day post-challenge	4 days post-challenge	6 days post-challenge	9 days post-challenge
Unchallenged control	100.0 \pm 0.0	100.0 \pm 0.0	98.3 \pm 0.6	97.9 \pm 0.6	97.1 \pm 1.4	99.7 \pm 0.3	99.3 \pm 0.7	95.1 \pm 1.9	99.2 \pm 0.6
<i>V. pectenica</i> 10 ⁶	28.9 \pm 4.1 *	0.0 \pm 0.0 *	Discontinued	Discontinued		96.1 \pm 0.9 *	41.2 \pm 2.8 *	33.4 \pm 2.1 *	21.8 \pm 2.4 *
<i>V. pectenica</i> 10 ⁵	98.2 \pm 0.6	1.6 \pm 1.3 *	Discontinued	Discontinued		97.7 \pm 0.7	86.6 \pm 3.5 *	85.2 \pm 1.6 *	75.7 \pm 1.4 *
<i>V. pectenica</i> 10 ⁴	98.9 \pm 0.5	60.7 \pm 9.8 *	21.5 \pm 4.8 *	Discontinued		98.9 \pm 0.5	95.5 \pm 1.1	94.6 \pm 1.2	91.0 \pm 1.5 *
<i>V. splendidus</i> 10 ⁶	92.1 \pm 1.7 *	87.4 \pm 1.6 *	79.9 \pm 2.5 *	81.0 \pm 1.9 *	75.5 \pm 4.0 *				
<i>V. splendidus</i> 10 ⁵	99.7 \pm 0.3	98.5 \pm 0.9	97.6 \pm 1.6	97.5 \pm 0.9	95.8 \pm 1.1				
<i>V. splendidus</i> 10 ⁴	98.9 \pm 0.5	98.7 \pm 0.7	99.3 \pm 0.4	97.5 \pm 1.1	95.4 \pm 1.0				

Table 3. Survival (% \pm S.E.) and size ($\mu\text{m} \pm$ S.E.) of scallop larvae throughout the larval period. Larvae were cultured together until the sixth day (post-fertilization), after which they were separated into separate tanks and provided probiotics or antibiotics. Values within a column not sharing a superscript are statistically different ($p < 0.05$). Columns without superscripts denote no statistical differences upon those days.

Treatment	d2	d4	d6	d8	d10	d12	d14	d16	d19	d21	d24	d27	d29
<i>Survival</i>													
No probiotic (control)	100	100	99.1 \pm 0.2	99.1 \pm 0.3	99.4 \pm 0.4 ^b	97.1 \pm 0.7	96.1 \pm 0.7 ^{abc}	92.8 \pm 1.3 ^{bc}	93.5 \pm 1.1 ^{abc}	89.2 \pm 2.3 ^{ab}	92.8 \pm 0.9	91.3 \pm 0.7 ^{ab}	91.4 \pm 0.9
Antibiotic				99.7 \pm 0.2	97.3 \pm 0.7 ^{ab}	96.1 \pm 0.9	91.2 \pm 1.6 ^b	87.8 \pm 1.3 ^c	88.1 \pm 1.3 ^c	87.9 \pm 1.7 ^b	88.3 \pm 1.1	85.1 \pm 1.9 ^b	85.0 \pm 2.6
<i>A. macleodii</i> 0444				99.2 \pm 0.4	98.3 \pm 0.6 ^{ab}	96.3 \pm 1.1	96.6 \pm 0.6 ^{ac}	94.3 \pm 1.0 ^{ab}	95.2 \pm 1.6 ^{ab}	93.3 \pm 0.8 ^{ab}	93.7 \pm 0.5	94.9 \pm 0.9 ^a	90.9 \pm 1.8
<i>Neptunomonas</i> sp. 0536				98.7 \pm 0.6	97.4 \pm 0.7 ^{ab}	98.6 \pm 0.5	98.1 \pm 0.8 ^a	96.8 \pm 0.8 ^{ab}	94.2 \pm 1.4 ^{abc}	94.9 \pm 1.2 ^a	92.4 \pm 2.7	93.0 \pm 1.1 ^{ab}	92.8 \pm 1.3
<i>P. gallaeciensis</i>				99.4 \pm 0.2	96.6 \pm 0.8 ^a	95.3 \pm 0.9	93.3 \pm 1.3 ^{bc}	92.7 \pm 1.1 ^{bc}	89.1 \pm 2.0 ^{bc}	92.7 \pm 1.1 ^{ab}	90.9 \pm 1.9	89.0 \pm 2.0 ^{ab}	92.9 \pm 1.8
<i>Pseudoalteromonas</i> sp. D41				99.1 \pm 0.4	98.1 \pm 0.6 ^{ab}	96.7 \pm 1.2	95.9 \pm 0.7 ^{abc}	96.0 \pm 1.3 ^{ab}	94.9 \pm 1.1 ^{ab}	92.0 \pm 1.7 ^{ab}	92.7 \pm 1.6	88.0 \pm 2.2 ^{ab}	84.0 \pm 3.3
Probiotic mix				99.5 \pm 0.3	98.4 \pm 0.5 ^{ab}	96.5 \pm 1.7	97.9 \pm 0.8 ^a	97.7 \pm 0.7 ^a	97.2 \pm 0.8 ^a	95.2 \pm 1.1 ^a	93.7 \pm 1.3	87.1 \pm 4.0 ^{ab}	91.0 \pm 1.1
<i>Shell size</i>													
No probiotic (control)								186.2 \pm 1.5 ^a			224.8 \pm 1.5 ^a	224.8 \pm 1.8 ^a	226.8 \pm 1.6 ^{ab}
Antibiotic								176.6 \pm 1.6 ^b			213.4 \pm 2.2 ^{bc}	218.2 \pm 2.1 ^{ab}	217.9 \pm 2.3 ^{cd}
<i>A. macleodii</i> 0444								184.9 \pm 1.2 ^{ac}			215.7 \pm 2.0 ^b	223.3 \pm 2.1 ^a	228.6 \pm 1.5 ^a
<i>Neptunomonas</i> sp. 0536								183.5 \pm 1.2 ^{abc}			208.0 \pm 2.0 ^{bc}	214.4 \pm 2.1 ^{bc}	218.8 \pm 1.6 ^{cd}
<i>P. gallaeciensis</i>								180.0 \pm 1.4 ^{bc}			206.3 \pm 2.3 ^c	224.1 \pm 1.9 ^a	220.4 \pm 1.8 ^{bc}
<i>Pseudoalteromonas</i> sp. D41								182.3 \pm 1.3 ^{abc}			208.6 \pm 2.2 ^{bc}	212.2 \pm 1.8 ^c	212.5 \pm 1.7 ^d
Probiotic mix								180.0 \pm 1.5 ^{bc}			215.3 \pm 1.9 ^{bc}	214.0 \pm 1.9 ^{bc}	214.2 \pm 1.6 ^{cd}

Figure 1. Yield of competent larvae (% \pm S.E.), i.e. proportion of larvae to reach metamorphosis capability. Figures show: probiotic use alone (A) and probiotic use against pathogen-challenge (B). Columns not sharing a superscript are statistically different at day 29 ($p < 0.05$).

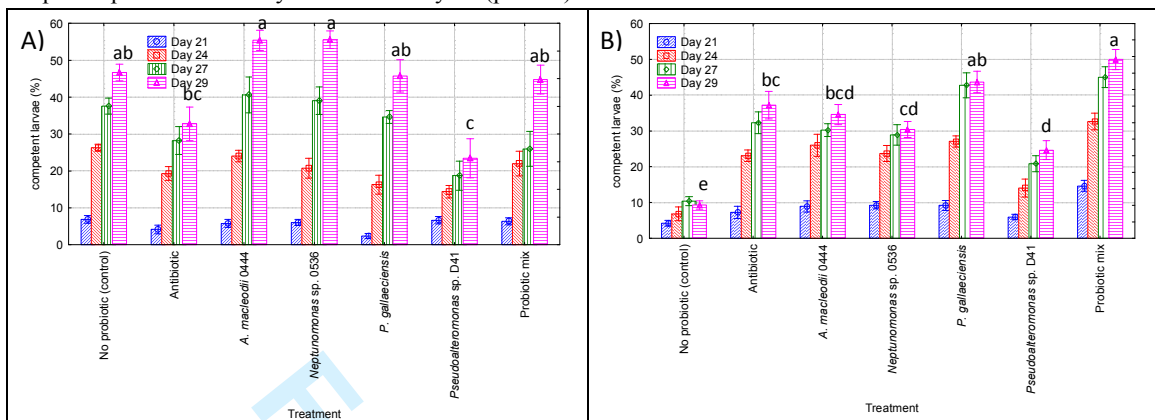


Table 4. Survival (% \pm S.E.) of scallop larvae throughout the larval period when challenged with pathogen, *V. pectenica*, on the seventh day. Larvae were cultured together until the sixth day, after which they were separated into separate tanks and provided probiotics or antibiotics. Values within a column not sharing a superscript are statistically different ($p < 0.05$).

Treatment	d2	d4	d6	d8	d10	d12	d14	d16	d19	d21	d24	d27	d29
<i>Survival</i>													
Pathogen control	100	100	99.1 \pm 0.2	99.2 \pm 0.5	87.4 \pm 1.0 ^b	72.9 \pm 3.2 ^c	63.2 \pm 1.7 ^c	52.6 \pm 3.0 ^d	31.6 \pm 2.6 ^d	30.1 \pm 2.4 ^c	23.6 \pm 2.0 ^d	22.9 \pm 2.1 ^d	20.3 \pm 2.9 ^c
Antibiotic				99.2 \pm 0.3	95.8 \pm 1.1 ^a	94.1 \pm 1.6 ^a	84.6 \pm 2.1 ^a	85.4 \pm 2.7 ^a	83.3 \pm 1.1 ^a	82.5 \pm 2.2 ^a	83.7 \pm 1.9 ^a	81.4 \pm 1.3 ^a	85.1 \pm 1.5 ^a
<i>A. macleodii</i> 0444				98.9 \pm 0.3	89.3 \pm 1.7 ^b	79.9 \pm 2.4 ^{bc}	69.9 \pm 1.8 ^c	63.0 \pm 2.2 ^{cd}	52.8 \pm 3.4 ^{bc}	45.0 \pm 1.7 ^b	46.9 \pm 2.8 ^{bc}	45.8 \pm 0.8 ^{bc}	43.9 \pm 2.7 ^b
<i>Neptunomonas</i> sp. 0536				99.1 \pm 0.4	89.9 \pm 1.7 ^b	81.1 \pm 2.2 ^{bc}	72.2 \pm 2.3 ^{bc}	70.4 \pm 2.1 ^{bc}	63.9 \pm 1.7 ^b	55.1 \pm 3.7 ^b	52.8 \pm 3.5 ^b	51.8 \pm 3.2 ^b	49.6 \pm 2.9 ^b
<i>P. gallaeciensis</i>				99.3 \pm 0.3	85.1 \pm 1.7 ^b	83.8 \pm 2.4 ^{bc}	79.6 \pm 2.8 ^{ab}	80.2 \pm 2.5 ^{ab}	79.9 \pm 3.0 ^a	76.3 \pm 3.6 ^a	78.3 \pm 1.8 ^a	81.4 \pm 1.6 ^a	79.6 \pm 2.5 ^a
<i>Pseudoalteromonas</i> sp. D41				99.6 \pm 0.4	82.8 \pm 2.4 ^b	80.2 \pm 2.7 ^{bc}	67.0 \pm 1.8 ^c	58.1 \pm 2.7 ^d	47.5 \pm 1.9 ^c	46.4 \pm 2.1 ^b	39.9 \pm 3.8 ^c	38.5 \pm 2.6 ^c	43.5 \pm 2.6 ^b
Probiotic mix				99.7 \pm 0.2	89.9 \pm 1.0 ^b	85.8 \pm 1.9 ^b	81.7 \pm 1.2 ^a	74.0 \pm 1.0 ^b	76.2 \pm 2.6 ^a	77.3 \pm 3.0 ^a	77.3 \pm 1.4 ^a	80.3 \pm 1.9 ^a	75.0 \pm 2.9 ^a