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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. pectenicida* 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. pectenicida*, 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

of a reliable product (avoiding seasonal fluctuations/spat collection inconsistencies) and stock enhancement through selective breeding.

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

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

2. Materials and methods

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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. pectenicida* or *V. splendidus* were used. Strains were revived
138 from –80 °C stores in Marine Broth (Difco) at appropriate temperatures: *A. macleodii*

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

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

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

2.4. Probiotic trials during scallop larval rearing

2.4.1. Probiotic trials

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

Following preparation for their use in experiments (24 h), each strain was administered at the highest possible concentration (Table 1), determined by the end-concentration of each probiotic culture. Furthermore, because the growth rates of probiotics differed (resulting in a range of end-concentrations), the final "in-tank" concentration of each probiotic ranged between 10^4-10^5 CFU ml^{-1} on the administration days. These concentrations were the same as those tested previously which demonstrated a positive probiotic effect upon scallop larvae (Kesarcodi-Watson et al., 2012).

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

TABLE 1.

2.4.2. Pathogen-challenge trials

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

2.5. Data analysis

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

3. Results

3.1. Age as a factor of scallop larvae pathogen susceptibility

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

TABLE 2

3.3. Probiotic trials

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

TABLE 3

FIGURE 1

3.4. Pathogen-challenge trials

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

TABLE 4

4. Discussion

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

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

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

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

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

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

Recent work in Norway developed low-exchange continuous-flow techniques for *P. maximus* larval rearing which allowed successful larval rearing in the absence of antibiotics (Andersen, Burnell & Bergh, 2000; Torkildsen & Magnesen, 2004; Magnesen et al., 2006; Andersen et al., 2013). During this work, a very low water turn-over (approximately one tank exchange per day) and low density of larvae (3.0-5.2 larvae ml^{-1} starting density, with a final density at settlement of 1.0 larvae ml^{-1}) were used (Magnesen et al., 2006, Andersen et al., 2013). The work in Norway lays a good foundation to antibiotic-free scallop larviculture (in an industry with increasing restrictions on antibiotic use), but with such low water exchange it currently remains a slightly more intensive version of a batch-culture system; improvements in stocking density and water-flow would be desirable to provide efficient larval rearing. To our knowledge, commercial rearing of scallop larvae by flow-through techniques is currently practiced only in Norway.

Continued research is needed into scallop larval rearing to facilitate regular and untroubled production. The results from this study provide useful information for direct application in scallop larviculture and also highlight further research avenues.

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References

Andersen, S., Burnell, G., Bergh, Ø., 2000. Flow-through systems for culturing great scallop larvae. Aquacult. Int. 8, 249-257.

Andersen, S., Christophersen, G., Magnesen, T., 2011. Spat production of the great scallop (*Pecten maximus*): a roller coaster. Can. J. Zool. 89, 579-598.

Andersen, S., Christophersen, G., Magnesen, T., 2013. Implications of larval diet concentration on post-larval yield in a production scale flow-through system for scallops (*Pecten maximus* Lamarck) in Norway. Aquacult. Int. 21, 435-452.

Avendaño, R.E., Riquelme, C.E., 1999. Establishment of mixed probiotics and microalgae as food for bivalve larvae. Aquac. Res. 30, 893-900.

Bergh, Ø., Naas, K.E., Harboe, T., 1994. Shift in intestinal microflora of Atlantic halibut (*Hippoglossus hippoglossus*) larvae during first feeding. Can. J. Fish. Aquat. Sci. 51, 1899-1903.

FAO Fisheries and Aquaculture Information and Statistics Service. 2010. Aquaculture production 1950-2008. FISHSTAT Plus – Universal software for fishery statistical time series [online or CD-ROM]. Food and Agriculture Organization of the United Nations. Available at: http://www.fao.org/fishery/statistics/software/fishstat/en

Helm, M.M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves. A practical manual. In: Lovatelli, A. (Ed.), FAO Fisheries Technical Paper 471. Food and Agriculture Organization of the United, Nations Publishing, Rome, Italy. 177 pp.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J., Gibson, L., 2008. Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture 272, 1-14.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J., Gibson, L., 2010. *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, two novel probiotics for hatchery-reared Greenshell™ mussel larvae, *Perna canaliculus*. Aquaculture 309, 49-55.

Kesarcodi-Watson, A., Miner, P., Nicolas, J.-L., Robert, R., 2012. Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). Aquaculture 344-349, 29-34.

Le Pennec, M., Prieur, D., Chardi, P., 1973. Développement larvaire de *Mytilus edulis* (L.) en presence d'antibiotiques. 2^{éme} partie: action sur la croissance de quatre antibiotiques: Auréomycine, Erythromycine, Chloramphenicol et Sulfamérazine. Revue Internationale d'Oceanographie Medicale 30, 115-137.

Magnesen, T., Bergh, Ø., Christophersen, G., 2006. Yields of great scallop, *Pecten maximus*, larvae in a commercial flow-through rearing system in Norway. Aquacult. Int. 14, 377-394.

Magnesen, T., Jacobsen, A., 2012. Effect of water recirculation on seawater quality and production of scallop (*Pecten maximus*) larvae. Aquacult. Eng. 47, 1-6.

Marty, Y., Delaunay, F., Moal, J. Samain, J.F., 1992. Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. J. Exp. Mar. Biol. Ecol. 163, 221-234.

Merino, G., Uribe, E., Soria, G., von Brand, E., 2009. A comparison of larval production of the northern scallop, *Argopecten purpuratus*, in closed and recirculating culture systems. Aquacult. Eng. 40, 95-103.

Pernet, F., Bricelj, V.M., Parrish, C.C., 2005. Effects of varying dietary levels of ω6 polyunsaturated fatty acids during the early ontogeny of the sea scallop, *Placopecten magellanicus*. J. Exp. Mar. Biol. Ecol. 327, 115-133.

Ragg, N.L.C., King, N., Watts, E., Morrish, J., 2010. Optimising the delivery of the key diatom *Chaetoceros calcitrans* to intensively cultured Greenshell™ mussel larvae, *Perna canaliculus*. Aquaculture 306, 270-280.

Riquelme, C., Hayashida, G., Toranzo, A.E., Vilches, J., Chavez, P.,1995. Pathogenicity studies on a *Vibrio anguillarum*-related (VAR) strain causing and epizootic in *Argopecten purpuratus* larvae cultured in Chile. Dis. Aquat. Organ. 22, 135-141.

Riquelme, C., Hayashida, G., Araya, R., Uchida, A., Satomi, M., Ishida, Y., 1996. Isolation of a native bacterial strain from the scallop *Argopecten purpuratus* with inhibitory effects against pathogenic vibrios. J. Shellfish Res. 15(2), 369-374.

Riquelme, C., Araya, R., Vergara, N., Rojas, A., Guaita, M., Candia, M., 1997. Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 154, 17-26.

Riquelme, C., Araya, R., Escribano, R., 2000. Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. Aquaculture 181, 25-36.

Riquelme, C.E., Jorquera, M.A., Rojas, A.I., Avendaño, R.E., Reyes, N., 2001. Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). Aquaculture 192, 111–119.

Robert, R., Gérard, A., 1999. Bivalve hatchery techniques: current situation for the oyster *Crassostrea gigas* and the scallop *Pecten maximus*. Aquat. Living Resour., 12 (2), 121-130.

Robert, R., Miner, P., Nicolas, J.L., 1996. Mortality control of scallop larvae in the hatchery. Aquacult. Int. 4, 305-313.

Robert, R., Nicolas, L., Moisan, C., Barbier, G. 1999. Caractérisation morphologique et biochimique de la metamorphose de la coquille St Jacques *Pecten maximus* (Lamarck). C.R. Acad. Sci. Paris 322, 847-853.

Ruiz-Ponte, C., Cilia, V., Lambert, C., Nicolas, J.L., 1998. *Roseobacter gallaeciensis* sp. nov.,a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*. Int. J. Syst. Bacteriol. 48, 537–542.

Ruiz-Ponte, C., Samain, J.F., Sánchez, J.L., Nicolas, J.L., 1999. The benefit of a *Roseobacter* species on the survival of scallop larvae. Mar. Biotechnol. 1, 52-59.

Sandaa, R.-A., Brunvold, L., Magnesen, T., Bergh, Ø., 2008. Monitoring the opportunistic bacteria *Pseudoalteromonas* sp. LT-13 in a great scallop, *Pecten maximus* hatchery. Aquaculture 276, 14-21.

485 Soria, G., Merino, G., von Brand, E., Uribe, E., 2007. Culturing northern Chilean
486 scallop *Argopecten purpuratus* larvae in closed and recirculating aquaculture
487 systems. J. Shellfish Res. 26, 1347-1348.

488 Soudant, P., Marty, Y., Moal, J., Masski, H., Samain, J.F., 1998. Fatty acid
489 composition of polar lipid classes during larval development of scallop *Pecten*
490 *maximus* (L.). Comp. Biochem. Phys. A 121, 279-288.

491 Torkildsen, L., Lambert, C., Nylund, A., Magnesen, T., Bergh, Ø., 2005. Bacteria
492 associated with early life stages of the great scallop, *Pecten maximus*: impact on
493 larval survival. Aquacult. Int. 13, 575-592.

494 Torkildsen, L., Magnesen, T., 2004. Hatchery production of scallop larvae (*Pecten*
495 *maximus*)- survival in different rearing systems. Aquacult. Int. 12, 489-507.

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
A. macleodii 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
Neptunomonas 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
P. gallaeciensis	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
Pseudoalteromonas 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	2.3×10^8	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

Table 2. Scallop larvae survival (% ± S.E.) after two separate pathogen-challenges with a range of concentrations. During the first challenge, *V. pectenicida* or *V. splendidus* was used. During the second challenge only *V. pectenicida* 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 asterix (*) 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 ± 0.0	100.0 ± 0.0	98.3 ± 0.6	97.9 ± 0.6	97.1 ± 1.4	99.7 ± 0.3	99.3 ± 0.7	95.1 ± 1.9	99.2 ± 0.6
V. pectenicida 10^6	28.9 ± 4.1 *	0.0 ± 0.0 *	Discontinued	Discontinued		96.1 ± 0.9 *	41.2 ± 2.8 *	33.4 ± 2.1 *	21.8 ± 2.4 *
V. pectenicida 10^5	98.2 ± 0.6	1.6 ± 1.3 *	Discontinued	Discontinued		97.7 ± 0.7	86.6 ± 3.5 *	85.2 ± 1.6 *	75.7 ± 1.4 *
V. pectenicida 10^4	98.9 ± 0.5	60.7 ± 9.8 *	21.5 ± 4.8 *	Discontinued		98.9 ± 0.5	95.5 ± 1.1	94.6 ± 1.2	91.0 ± 1.5 *
V. splendidus 10^6	92.1 ± 1.7 *	87.4 ± 1.6 *	79.9 ± 2.5 *	81.0 ± 1.9 *	75.5 ± 4.0 *				
V. splendidus 10^5	99.7 ± 0.3	98.5 ± 0.9	97.6 ± 1.6	97.5 ± 0.9	95.8 ± 1.1				
V. splendidus 10^4	98.9 ± 0.5	98.7 ± 0.7	99.3 ± 0.4	97.5 ± 1.1	95.4 ± 1.0				

Table 3. Survival (% ± S.E.) and size (µm ± 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
Survival													
No probiotic (control)	100	100	99.1 ± 0.2	99.1 ± 0.3	99.4 ± 0.4 ^{b}	97.1 ± 0.7	96.1 ± 0.7 ^{abc}	92.8 ± 1.3 ^{bc}	93.5 ± 1.1 ^{abc}	89.2 ± 2.3 ^{ab}	92.8 ± 0.9	91.3 ± 0.7 ^{ab}	91.4 ± 0.9
Antibiotic				99.7 ± 0.2	97.3 ± 0.7 ^{ab}	96.1 ± 0.9	91.2 ± 1.6 ^{b}	87.8 ± 1.3 ^{c}	88.1 ± 1.3 ^{c}	87.9 ± 1.7 ^{b}	88.3 ± 1.1	85.1 ± 1.9 ^{b}	85.0 ± 2.6
A. macleodii 0444				99.2 ± 0.4	98.3 ± 0.6 ^{ab}	96.3 ± 1.1	96.6 ± 0.6 ^{ac}	94.3 ± 1.0 ^{ab}	95.2 ± 1.6 ^{ab}	93.3 ± 0.8 ^{ab}	93.7 ± 0.5	94.9 ± 0.9 ^{a}	90.9 ± 1.8
Neptunomonas sp. 0536				98.7 ± 0.6	97.4 ± 0.7 ^{ab}	98.6 ± 0.5	98.1 ± 0.8 ^{a}	96.8 ± 0.8 ^{ab}	94.2 ± 1.4 ^{abc}	94.9 ± 1.2 ^{a}	92.4 ± 2.7	93.0 ± 1.1 ^{ab}	92.8 ± 1.3
P. gallaeciensis				99.4 ± 0.2	96.6 ± 0.8 ^{a}	95.3 ± 0.9	93.3 ± 1.3 ^{bc}	92.7 ± 1.1 ^{bc}	89.1 ± 2.0 ^{bc}	92.7 ± 1.1 ^{ab}	90.9 ± 1.9	89.0 ± 2.0 ^{ab}	92.9 ± 1.8
Pseudoalteromonas sp. D41				99.1 ± 0.4	98.1 ± 0.6 ^{ab}	96.7 ± 1.2	95.9 ± 0.7 ^{abc}	96.0 ± 1.3 ^{ab}	94.9 ± 1.1 ^{ab}	92.0 ± 1.7 ^{ab}	92.7 ± 1.6	88.0 ± 2.2 ^{ab}	84.0 ± 3.3
Probiotic mix				99.5 ± 0.3	98.4 ± 0.5 ^{ab}	96.5 ± 1.7	97.9 ± 0.8 ^{a}	97.7 ± 0.7 ^{a}	97.2 ± 0.8 ^{a}	95.2 ± 1.1 ^{a}	93.7 ± 1.3	87.1 ± 4.0 ^{ab}	91.0 ± 1.1
Shell size													
No probiotic (control)								186.2 ± 1.5 ^{a}			224.8 ± 1.5 ^{a}	224.8 ± 1.8 ^{a}	226.8 ± 1.6 ^{ab}
Antibiotic								176.6 ± 1.6 ^{b}			213.4 ± 2.2 ^{bc}	218.2 ± 2.1 ^{ab}	217.9 ± 2.3 ^{cd}
A. macleodii 0444								184.9 ± 1.2 ^{ac}			215.7 ± 2.0 ^{b}	223.3 ± 2.1 ^{a}	228.6 ± 1.5 ^{a}
Neptunomonas sp. 0536								183.5 ± 1.2 ^{abc}			208.0 ± 2.0 ^{bc}	214.4 ± 2.1 ^{bc}	218.8 ± 1.6 ^{cd}
P. gallaeciensis								180.0 ± 1.4 ^{bc}			206.3 ± 2.3 ^{c}	224.1 ± 1.9 ^{a}	220.4 ± 1.8 ^{bc}
Pseudoalteromonas sp. D41								182.3 ± 1.3 ^{abc}			208.6 ± 2.2 ^{bc}	212.2 ± 1.8 ^{c}	212.5 ± 1.7 ^{d}
Probiotic mix								180.0 ± 1.5 ^{bc}			215.3 ± 1.9 ^{bc}	214.0 ± 1.9 ^{bc}	214.2 ± 1.6 ^{cd}

Figure 1. Yield of competent larvae (% ± 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 ($p<0.05$).

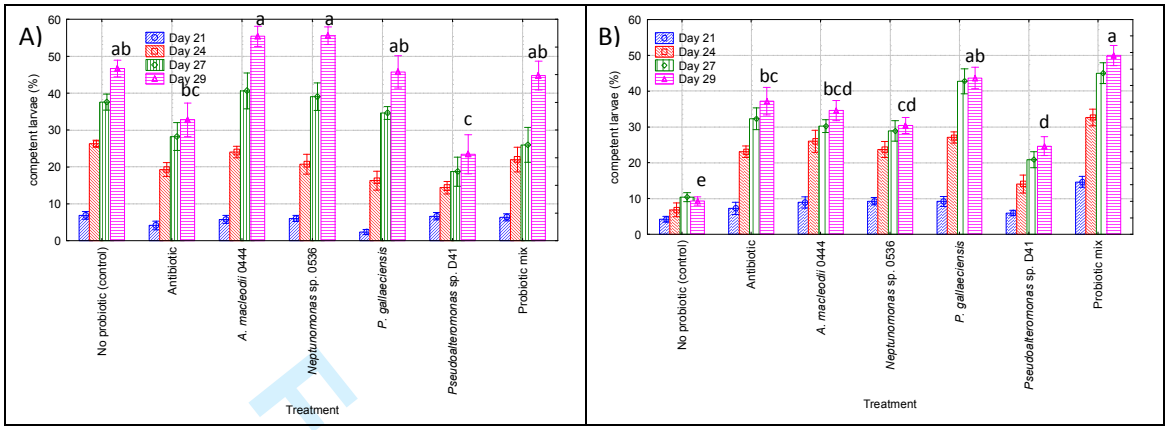

Table 4. Survival (% ± S.E.) of scallop larvae throughout the larval period when challenged with pathogen, *V. pectenicida*, on the seventh day. Larvae were cultured together until the sixth day, after which they were separated into separate tanks and provided probiotics or antibiotics. Values within 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
Survival													
Pathogen control	100	100	99.1 ± 0.2	99.2 ± 0.5	87.4 ± 1.0 ^{b}	72.9 ± 3.2 ^{c}	63.2 ± 1.7 ^{c}	52.6 ± 3.0 ^{d}	31.6 ± 2.6 ^{d}	30.1 ± 2.4 ^{c}	23.6 ± 2.0 ^{d}	22.9 ± 2.1 ^{d}	20.3 ± 2.9 ^{c}
Antibiotic				99.2 ± 0.3	95.8 ± 1.1 ^{a}	94.1 ± 1.6 ^{a}	84.6 ± 2.1 ^{a}	85.4 ± 2.7 ^{a}	83.3 ± 1.1 ^{a}	82.5 ± 2.2 ^{a}	83.7 ± 1.9 ^{a}	81.4 ± 1.3 ^{a}	85.1 ± 1.5 ^{a}
A. macleodii 0444				98.9 ± 0.3	89.3 ± 1.7 ^{b}	79.9 ± 2.4 ^{bc}	69.9 ± 1.8 ^{c}	63.0 ± 2.2 ^{cd}	52.8 ± 3.4 ^{bc}	45.0 ± 1.7 ^{b}	46.9 ± 2.8 ^{bc}	45.8 ± 0.8 ^{bc}	43.9 ± 2.7 ^{b}
Neptunomonas sp. 0536				99.1 ± 0.4	89.9 ± 1.7 ^{b}	81.1 ± 2.2 ^{bc}	72.2 ± 2.3 ^{bc}	70.4 ± 2.1 ^{bc}	63.9 ± 1.7 ^{b}	55.1 ± 3.7 ^{b}	52.8 ± 3.5 ^{b}	51.8 ± 3.2 ^{b}	49.6 ± 2.9 ^{b}
P. gallaeciensis				99.3 ± 0.3	85.1 ± 1.7 ^{b}	83.8 ± 2.4 ^{bc}	79.6 ± 2.8 ^{ab}	80.2 ± 2.5 ^{ab}	79.9 ± 3.0 ^{a}	76.3 ± 3.6 ^{a}	78.3 ± 1.8 ^{a}	81.4 ± 1.6 ^{a}	79.6 ± 2.5 ^{a}
Pseudoalteromonas sp. D41				99.6 ± 0.4	82.8 ± 2.4 ^{b}	80.2 ± 2.7 ^{bc}	67.0 ± 1.8 ^{c}	58.1 ± 2.7 ^{d}	47.5 ± 1.9 ^{c}	46.4 ± 2.1 ^{b}	39.9 ± 3.8 ^{c}	38.5 ± 2.6 ^{c}	43.5 ± 2.6 ^{b}
Probiotic mix				99.7 ± 0.2	89.9 ± 1.0 ^{b}	85.8 ± 1.9 ^{b}	81.7 ± 1.2 ^{a}	74.0 ± 1.0 ^{b}	76.2 ± 2.6 ^{a}	77.3 ± 3.0 ^{a}	77.3 ± 1.4 ^{a}	80.3 ± 1.9 ^{a}	75.0 ± 2.9 ^{a}