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Effect of the probiotic strain *Phaeobacter gallaeciensis* after bacterial challenge on the complete larval development of *Pecten maximus*

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Abstract – The aim of this project was to evaluate the impact of probiotic (*Phaeobacter gallaeciensis*, X34 strain) treatment on the complete development (from veliger to metamorphosis) of *Pecten maximus* larvae in the context of a bacterial challenge and in conditions more representative of hatchery practices. To that effect, the present study was divided into two main steps. In the first, we used in vitro analyses (antibiograms and microplate assays) to validate the inhibition abilities of X34 on the growth of four *Vibrio* pathogen species. During the second step, we added pathogens (*Vibrio pectenicida*) into rearing tanks after two weeks of pre-treatment with the probiotic and then followed the larval development of *Pecten maximus* through the monitoring of survival rates, shell lengths and metamorphosis ability. Moreover, antioxidant (catalase and superoxide dismutase) and lipids peroxidation activities were also measured after bacterial challenge in order to evaluate the physiological response of larvae to pathogen exposition. Our results indicated an activation of the two selected antioxidant enzymes after bacterial challenge, but the increase was significantly lower in probiotic treated larvae. At the end of the experiment, the strain X34 treatment prevented a mass mortality event and showed a significant increase in the number of individuals reaching competence, when compared to untreated larvae.

Keywords: Probiotics / hatchery / bacterial challenge / larval development / *Pecten maximus* / *Phaeobacter gallaeciensis* / *Vibrio*

1 Introduction

One of the major constraints affecting the seed production in bivalve hatcheries is the occurrence of high mortality due to the proliferation of opportunistic pathogens. Since the early beginnings of bivalve aquaculture, bacterial infections have been reported, particularly during larval stages (Paillard et al. 2004; Elston and Ford 2011). Most of these pathogenic bacteria can be traced back to *Vibrio* genera that regularly induce mass mortality events, thus causing major economic losses in this industry (Verschuere et al. 2000; Elston 2008; Elston and Ford 2011). Consequently, sanitary methods are commonly used to prevent the introduction of pathogens in hatcheries, among which quarantining the broodstock, chlorine treatment, heat, filtration, ozone, and ultraviolet irradiation (Jorquera et al. 2001; Elston and Ford 2011). To improve larval survival, antibiotics are also currently utilized for some bivalves including scallops (Pectinidae) and flat oysters (Ostreidae) to disinfect broodstock, to manage the bacterial load and to eliminate *Vibrio* (Robert et al. 1996; Nicolas et al. 1996; Torkildsen et al. 2000). However, antibiotic treatment is costly and can be hazardous to animals and human health, especially when since bacterial strains can develop resistance to the treatment (Cabello 2006). Probiotics have been proposed as a promising alternative to antibiotic treatment in bivalve hatcheries (Verschuere et al. 2000; Balcázar et al. 2006; Kesarcodi-Watson et al. 2008; Prado et al. 2010). Although the probiotics display less antibacterial antagonism than antibiotics they could exert other beneficial aspects such as growth enhancement and stimulation of the immune system (Sun et al. 2010; Granados-Amores et al. 2012).

As described by Verschuere et al. (2000), the selection of probiotic strains involves a multistep process comprising the screening of probiotic candidates by in vitro tests followed by experimental challenge with a known pathogen to establish the protecting efficiency in the host. For the larval production of bivalves, probiotics screening has so far essentially been performed using in vitro analyses (i.e. antibiograms, double-layer methods and microtiter plate assays) to evaluate the ability of candidate strains to inhibit growth of known pathogens

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(Riquelme et al. 1996, 2001; Prado et al. 2009). These experiments were generally followed by in vivo validation of probiotic effects of the selected strains by assessing the improvement in larval survival although this was usually done in the absence of a bacterial challenge. Recently, several studies have developed in vivo validation protocols for probiotics using bacterial challenges in small-scale bioassays (Kesarcodi et al. 2009, 2010, 2012; Karim et al. 2013), but these conditions remain very different from those of a commercial production in terms of water volume, larval densities and hatchery routines. Therefore, in this study, we aimed to evaluate the probiotic potential of the "X34" strain of *Phaeobacter gallaeciensis* on the entire larval development (from veliger stages to metamorphosis) of *P. maximus*, using a bacterial challenge experiment in conditions closer to those used in hatchery. Furthermore, in order to validate the efficiency of the larvae's response to the bacterial challenge, we measured the activation of antioxidant processes as an indicator of the physiological response of larvae to bacterial infection, as proposed by Genard et al. (2011, 2013). We test the hypothesis that a probiotics pre-treatment of several days (to allow colonization of larvae by the probiotics) protect *P. maximus* larvae against *Vibrio* pathogen and decrease the antioxidant responses of larvae in contact to pathogen. We test also the effect of probiotic treatment during all the larval rearing on the survival, growth and competence of larvae at the pediveliger stage.

2 Materials and methods

2.1 Bacterial strains

The bacterial strains used in this project were isolated from aquaculture environments. The four *Vibrio* strains (*V. aestuarianus* (LPI 02/041), *V. coralliilyticus* (LPI 06/210), *V. splendidus* (LPI 06/001) and *V. pectenicida* (LPI 01/006)) used for the in vitro experiments and bacterial challenge were isolated from diseased bivalve larvae and identified by sequencing of the 16S rRNA gene (Kesarcodi et al. 2012) while the probiotic strain of *Phaeobacter gallaeciensis* (X34) was extracted from larval tanks in bivalve hatcheries (Ruiz-Ponte et al. 1999). Stock cultures of these strains were stored at −80 °C in 20% (vol/vol) glycerol mixed with Marine Broth medium (Difco). Prior to their use in experiments, strains were streaked onto marine agar gel to ensure purity, and then cultured in Marine Broth under agitation (180 rpm) at 20 °C for 48 h for X34 and 24 h for pathogens. Optical density was read at 600 nm to assess bacterial concentration.

2.2 In vitro analyses of X34 inhibition abilities

Two in vitro analyses were performed to validate inhibition abilities of X34 strain against the development of selected pathogens. The first analysis consisted of an antibiogram-like method where the evaluation of the growth inhibition of pathogens around X34 is taken as evidence of antimicrobial activity. For this, Petri dishes containing Marine agar (Difco) were streaked with Marine Broth inoculated (10^5 cells ml^{-1}) with pathogen. Six wells (5 mm) were made in the agar and filled with 50 μl of X34 strains (10^5 cells ml^{-1}). Inhibitory ability was indicated by the presence of an inhibited growth ring of the pathogenic strain on the agar and the inhibition distance (mm) was measured from the border of the well to the edge of the clear zone after 48 h of incubation at 20 °C. Analyses were conducted in triplicate.

In the second analysis, microtiter plate assays were performed to assess if the inhibitory effect measured by the antibiogram-like method was associated with the release of antimicrobial compounds by the probiotic bacteria in their environment. Here, Marine Broth was inoculated and incubated with X34 strains, then the culture medium was centrifuged (12 000 g, 10 min, at 20 °C) and the resultant supernatants were removed and filter-sterilized through a 0.20 μm-pore size sterile filter (Whatman). Simultaneously, new batches of Marine Broth inoculated with pathogens were produced then diluted to reach a final concentration of 10^5 cells ml^{-1}. This pathogen, 100 μl, inoculated medium were distributed in 96-microplate wells filled with an equal volume of either X34 supernatant or Marine Broth as control. A mixture composed of X34 supernatant and bacteria-free Marine Broth was used as a blank. After a 24 h incubation at 20 °C, the inhibition effect of the X34 supernatant was estimated by comparing the optical densities of pathogen inoculated medium and the controls. The experiment was carried out on each pathogen strain in triplicate. Optical density (600 nm) was read using a microplate reader (Bioteck Instruments Inc., USA).

2.3 Rearing procedures and experimental design

This project was conducted at the IFREMER hatchery in Brest (Brittany, France). *P. maximus* broodstocks were collected at the experimental aquaculture station of Argenton (Brittany, France). Spawning was artificially induced by thermal shock and the fertilized eggs were transferred for incubation in 150 L cylindrical conical-based tanks for 24 h at 20 °C (10 eggs ml^{-1}) and provided with airflow. After 48 h, larvae (20 ind. ml^{-1} initially) were reared in triplicate at 20 °C in UV-treated filtered seawater (double cartridge-filtered: 1 μm and 0.5 μm) with gentle aeration during 14 days. During this period, larvae were treated either with probiotic, antibiotic (chloramphenicol at 4 mg ml^{-1}; Fluka) or left untreated (control). This pre-treatment period was performed to allow colonization of larvae by the probiotic. The antibiotic was used in this study to compare the efficiency of the probiotic's effect to antibiotic treatment. To avoid possible toxicity associated with X34 treatment, previous experiments were carried out to determine the optimal concentration of the probiotic and a concentration of 10^6 cells ml^{-1} was retained. Larvae were fed a mixture of *Pavlova lutheri*, *Isochrysis affinis galbana* and *Skeletonema costatum* at 20×10^3 algal cells ml^{-1} for both flagellates and 10×10^3 algal cells mL^{-1} for the diatom. After pre-treatment period, larvae were collected using a 100 μm sieve, divided into batches, and placed into 10 L containers at the concentration of 10 larvae ml^{-1}. At this time (16 dpf), a unique bacterial challenge using *V. pectinicida* was performed on half of these sieved larvae. Others larvae were continued to be supplied with probiotics, antibiotics (in the same concentrations as for the pre-treatment period) or left untreated. Each combination of

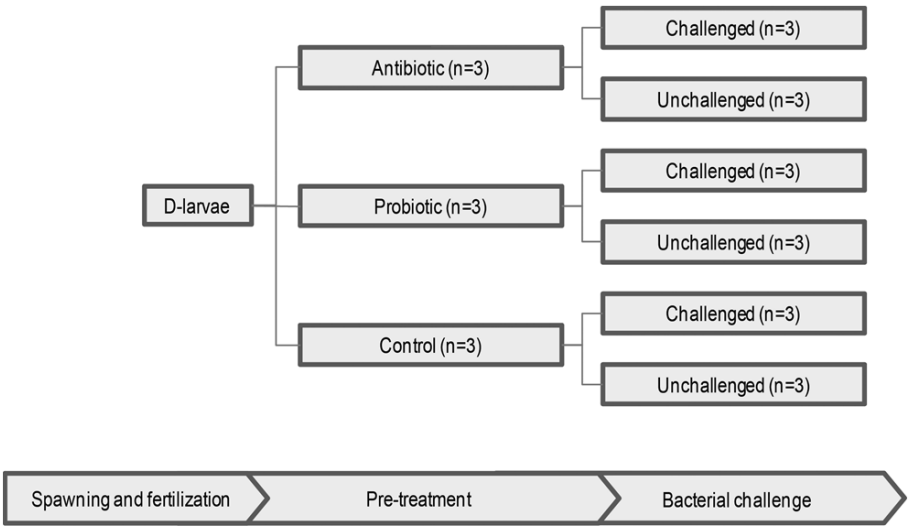

Fig. 1. Experimental design of bacterial challenge (*Vibrio pectenicida*) experiment on *Pecten maximus* larvae. After spawning and eggs fertilization, D-larvae were sorted and divided equally into three treatments (antibiotic, probiotic and untreated or control). A pre-treatment period of ten days was carried out before bacterial challenge in order to allow probiotic colonization of the larvae. Larvae were cultivated in triplicate ($n = 3$).

treatments was tested in triplicate (see Fig. 1 for design). Water renewal, probiotic addition and antibiotic treatment were conducted every 2–3 days while microalgae were distributed every day. Bathing challenge was performed at the concentration of 10^5 bacterial cells ml^{-1}. Experiments were conducted from the veliger stages until metamorphosis.

2.4 Larval development

The effect of probiotic treatment on larval development of scallop larvae was estimated through larval growth, survival rate and assessment of metamorphosis ability. Shell lengths and survival rates were monitored at each water renewal. Three 1 ml subsamples of sieved larvae were removed and the concentration of live larvae was determined visually using a binocular microscope (Leitz Labovert, Wetzlar, Germany). Shell length was estimated with a Moticam 320 camera coupled to the binocular microscope. At least, 50 live larvae per tank were measured to determine the mean shell length using Motic Images version 2.0 (DC Imaging, USA). The mortality rates were estimated visually on formaldehyde treated samples of 200 to 300 individuals using an optical microscope and a counting plate and expressed as percentages (% of live larvae based on initial number). At the end of the pediveliger stage and the onset of metamorphosis, a double ring can be observed on the shells of larvae and this is considered as a reliable criterion expressing the larvae's capability to undergo metamorphosis (Robert and Gérard 1999). The double-ringed individuals were counted visually and expressed as percentages (% of double-ringed larvae based on live larvae).

2.5 Antioxidant analyses

Several spectrophotometric analyses were used to investigate oxidative stress through the activation of two enzymes associated to antioxidant defences (superoxide dismutase and catalase) and through the level of lipids peroxidation (malondialdehyde, MDA). All of these analyses were performed using a microplate reader (Bioteck Instruments Inc., USA) with methods described in Genard et al. (2011). Briefly, samples were homogenized on ice in phosphate buffered saline (PBS) containing 0.1% of triton X-100. After centrifugation of the homogenates, the supernatants were collected and stored at −80 °C. The protein contents were measured on homogenate colorimetrically as described by Lowry et al. (1968). The superoxide dismutase (SOD, EC 1.15.1.1) activity was measured spectrophotometrically according to the methods of McCord and Fridovich (1969) and Flohé and Ötting (1985) modified for small volumes in a microplate reader. In the latter methods, inhibition of the cytochrome c reduction rate is monitored at 550 nm utilizing the hypoxantine/xantine oxidase (XOD) system as the source of superoxide ($O2^{\circ}$). CAT (CAT, EC 1.11.1.6) activity was determined using the Amplex® Red Catalase Assay Kit (Invitrogen, USA) according to the manufacturer's protocol. This commercial kit uses the horseradish peroxidase (HRP)-catalyzed oxidation of 9-acetylresorufin to fluorescent resorufin with a maximal emission at 587 nm. Oxidative stress was investigated trough the lipid peroxidation levels using the malondialdehyde (MDA) content of larvae. MDA was measured with BIOXYTECHO MDA-586TM assay kits (Oxisresearch, USA). This method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with MDA monitored at 586 nm. Except for SOD where one unit of activity was defined as the amount of enzyme that inhibits the rate of cytochrome C reduction by 50%, enzyme activity was reported in quantity (in pmol) of substrate transformed per minute, expressed per larva or ng of protein.

2.6 Statistical analysis

Analyses were carried out using SAS® software (version 8.2). The significance value for all analyses was set at

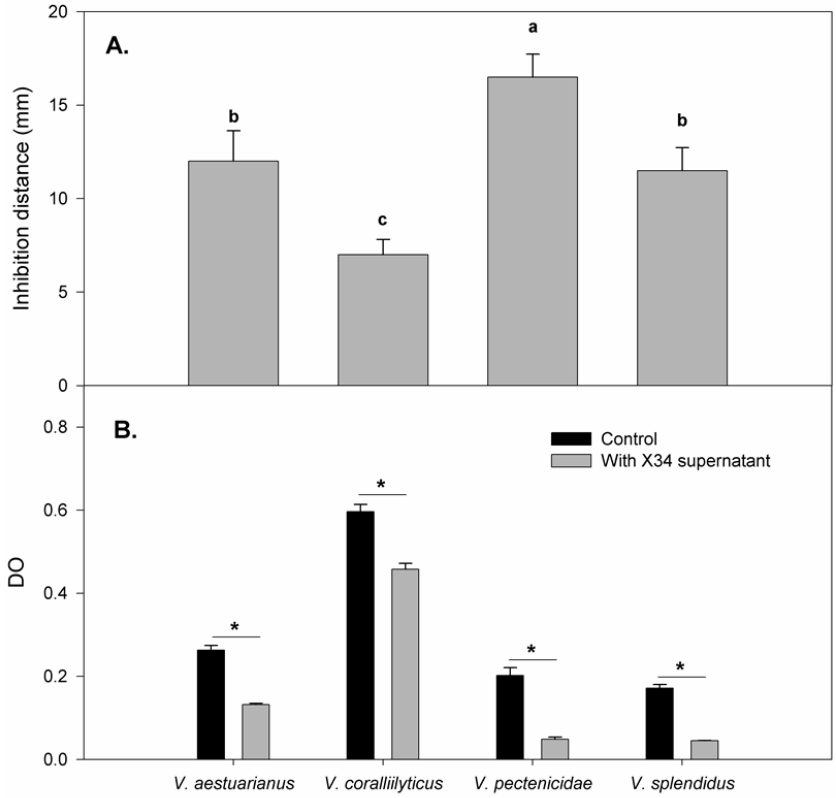

Fig. 2. Inhibitory ability of *Phaeobacter gallaeciensis* (X34 strain) on growth of four *vibrio* pathogen species (*Vibrio aestuarianus, V. coralliilyticus V. splendidus* and *V. pectinicida*) using antibiogram (A) and microplate (B) assays. For antibiogram tests the inhibitory ability was indicated as the inhibition distance (mm) measured from the border of the well to the edge of the clear zone after 48 h of incubation at 20 °C. In microplate assays, the inhibitory effect on pathogen growth was estimated by comparing optical density (OD) between wells containing the pathogen reared in supernatants of probiotics culture medium or Marine Broth. Data are means ± SD of three replicates. Different letters or * indicate significant differences.

$p < 0.05$. Student tests were performed to compare inhibition distances (antibiogram tests) and optical densities (microplate analysis). ANOVA (GLM) was used to evaluate treatment effect on survival and growth at the end of the pre-treatment period. After bacterial challenge experiments, a two-ways ANOVA (GLM) with repeated measures (time) was used to determine whether the effects of time, treatment (probiotic, antibiotic or untreated) and interaction of both were significant in challenged and unchallenged larvae on growth, survival and double ring rates. When differences were detected, LSMEANS a posteriori comparison tests were used. Residuals were plotted for assessment of normality. Homogeneity of variances was tested using the O'Brien test. Survival rates were normalized using angular transformation (Arcsin of root square). Data are presented as means ± standard error.

3 Results

3.1 In vitro validation of X34 probiotic abilities

In both in vitro experiments used in this project, we observed that X34 exhibited real inhibition abilities on growth of the four pathogen strains tested. In the antibiogram approach, the highest inhibition values were recorded for *V. pectinicida*, followed by *V. aestuarianus*, *V. splendidus* and finally *V. coralliilyticus* (Fig. 2A). The second set of analyses was carried out to evaluate if these inhibitory abilities were associated with the production of bactericidal compounds which are effective in a liquid medium (microplate analyses). Consistently with the results of the antibiogram tests, growth inhibitions were measured for the four pathogens tested. When compared to the controls, significantly lower OD values were found when pathogen inoculates were mixed with X34 medium supernatants (Fig. 2B).

3.2 Bacterial challenge, probiotic treatment and larval development

Before bacterial challenges, larvae were treated either with the probiotic, the antibiotic or they were left untreated during two weeks. At the end of this pre-treatment period, no significant differences between treatments were observed in term of survival rates and shell lengths (survival: $p = 0.059$, shell length: $p = 0.051$) and the averaged values obtained for these two variables were 95 ± 1% for survival rate and 179 ± 13 μm for shell length.

After this pre-treatment period, the impact of the bacterial challenge on larval development was only observed in survival

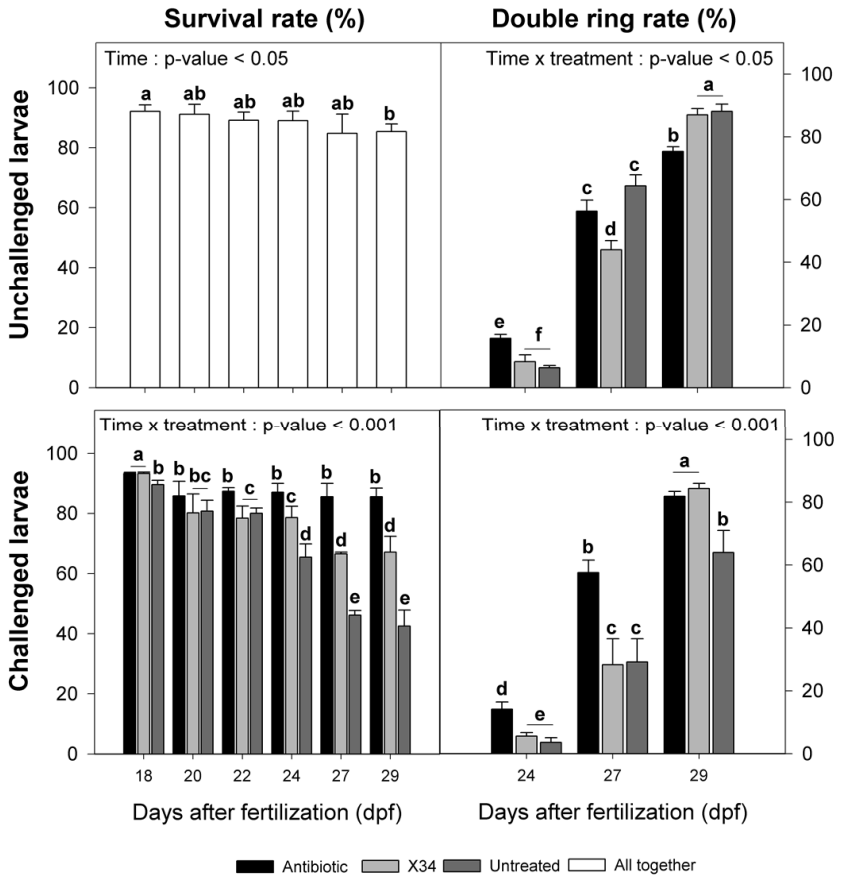

Fig. 3. Percentage of survival and double ring rates of *Pecten maximus* larvae challenged or not with *V. pectinicida* as a function of development time (days post fertilization, dpf) and larval treatment. Larvae were treated with antibiotic, probiotic (X34 stain) or untreated. Data from different treatments were pooled when this effect was not significant. Data are means ± SD of three replicate tanks. Different letters indicate significant differences.

and double ring rates, as similar shell length were recorded independently of challenge and treatment (shell length at 29 dpf, 220 ± 24 μm). Six days after pathogen inoculation (22 dpf), significantly lower survival rates were observed in probiotic and untreated larvae comparatively to antibiotic treated ones which remained constant until the end of the experiment (Fig. 3). At 24 dpf, in comparison to the probiotic treatment, lower survival rates were measured in untreated larvae. This difference increased in time until 29 dpf, where values of 67 and 43% were observed in X34 treated and untreated larvae respectively (Fig. 3). The bacterial challenge also affected the metamorphosis abilities of larvae. At 27 dpf, we observed significantly lower double ring rates in X34 treated and untreated larvae comparatively to the antibiotic treatment. Finally, at 29 dpf, similar rates (~83%) were found in antibiotic and X34 treated larvae while this rate was approximately 23% lower in untreated ones (Fig. 3).

This pattern was different when larvae were not submitted to a *Vibrio* challenge. In unchallenged larvae, we observed that survival rates did not change significantly between the three treatments (antibiotic, probiotic and untreated larvae) and reached values approximately 85% after 29 dpf (Fig. 3), while for metamorphosis ability, the double ring rates increased significantly from 24 to 29 dpf in all treatments (Fig. 3). Furthermore, the antibiotic treated larvae exhibited significantly lower ratios of double-ringed larvae at 29 dpf comparatively to X34 treated and untreated larvae (Fig. 3).

3.3 Bacterial challenge and oxidative stress

Antioxidant response and oxidative stress were investigated 48 h after pathogen inoculation. Our results indicated an activation of the two selected antioxidant enzymes (SOD and CAT) after bacterial challenge (Table 1). In CAT, the activity increased with the bacterial challenge in both X34 and untreated larvae, but not in antibiotic treatment. However, this increase was significantly higher in untreated larvae comparatively to probiotic treated larvae. A similar pattern was found in SOD activities where significantly higher activities were observed in challenged X34 and untreated larvae (Table 1). As observed in CAT activities, the most important difference was recorded in untreated larvae where the activity was six times higher in challenged individuals while this change was approximately 3.5 fold in X34 larvae (Table 1). Beside the activation of antioxidant defences after the bacterial challenge, we did not measure significant differences in peroxidation levels as illustrated by MDA concentrations, suggesting no oxidative stress was associated with the bacterial challenge (Table 1).

Table 1. Summary of antioxidant activities (catalase CAT, and superoxide dismutase SOD) and lipid peroxidation levels (malondialdehyde MDA) of *Pecten maximus* larvae challenged or not with *Vibrio pectenicida* as a function of larval treatment. Larvae were treated with antibiotic, probiotic (X34 strain) or untreated.

Antioxidant features	Unit	Antibiotic		X34		Untreated		Two-ways ANOVA (*p-value*)		
		Challenged	Unchallenged	Challenged	Unchallenged	Challenged	Unchallenged	Treatment	Challenge	Interaction
CAT	pmol larva^{-1} min^{-1}	49.5 ± 2.4 (c)	37.7 ± 2.9 (c)	105.0 ± 10.1 (b)	41.4 ± 6.1 (c)	144.0 ± 5.6 (a)	36.2 ± 1.8 (c)	<0.0001	<0.0001	<0.0001
	pmol ng^{-1} protein min^{-1}	2.1 ± 0.3 (c)	1.4 ± 0.2 (c)	3.9 ± 0.8 (b)	1.7 ± 0.5 (c)	5.6 ± 0.5 (a)	1.5 ± 0.1 (c)	<0.0001	<0.0001	0.001
SOD	μU larva^{-1} min^{-1}	158.4 ± 15.9 (c)	126.6 ± 36.5 (c)	532.1 ± 151.1 (b)	155.7 ± 20.2 (c)	870.7 ± 162.2 (a)	146.7 ± 57.9 (c)	0.001	<0.0001	0.001
	μU ng^{-1} protein min^{-1}	9.1 ± 1.9 (c)	4.2 ± 1.2 (d)	17.7 ± 5.0 (b)	5.2 ± 0.7 (d)	29.0 ± 5.4 (a)	3.6 ± 2.6 (d)	0.001	<0.0001	0.001
MDA	pmol larva^{-1}	0.08 ± 0.01 (a)	0.08 ± 0.01 (a)	0.07 ± 0.01 (a)	0.09 ± 0.01 (a)	0.07 ± 0.01 (a)	0.08 ± 0.01 (a)	0.303	0.097	0.423
	pmol μg^{-1} protein	7.1 ± 1.9 (a)	7.6 ± 0.8 (a)	9.3 ± 4.3 (a)	7.5 ± 2.7 (a)	6.7 ± 1.4 (a)	7.7 ± 1.2 (a)	0.629	0.949	0.548

Data are means ±SD of three replicate tanks. Significant values in two-ways ANOVA tests are in bold and different letters indicate significant differences.

4 Discussion

The in vitro analyses confirmed that *P. gallaeciensis* (X34) exhibited interesting inhibitory abilities against the four *Vibrio* pathogens tested. Antibacterial activities associated with this *Phaeobacter* species had already been reported in previous works aiming to select potential probiotics for bivalve hatcheries (Ruiz-Ponte et al. 1999; Prado et al. 2009; Kersarcodi-Watson et al. 2012; Karim et al. 2013). Our results confirm the promising probiotic potential of this strain against pathogens regularly encountered in hatcheries. The probiotic concentration retained for in vivo experiment was 10^6 cells ml^{-1} because higher concentrations induced mortality (data not shown). This concentration was in accordance with previous works which determined optimums for probiotic treatment between 10^4 and 10^6 cells ml^{-1} (Jeanthon et al. 1988; Ruiz-Ponte et al. 1999). The microplate assays indicate that the antibacterial effect is likely associated with the production of antibacterial compounds released by the bacteria in the environment, as suggested by Ruiz-Ponte et al. (1999). The exact nature of these compounds was not investigated in the present study and should be the subject of future research. Nevertheless, *P. gallaeciensis* strains are known to produce broad-spectrum antibiotics, tropodithietic acid (TDA) and its valence tautomer thiotropocin (Geng et al. 2008; Berger et al. 2011).

We confirm our hypothesis on the protecting effect of X34 probiotic on *P. maximus* larvae exposed to *Vibrio pectenicida*, when larvae were pre-treated with the probiotic. We suggest that TDA released by *P. gallaeciensis* limited pathogen proliferation and the occurrence of subsequent mortality events, explaining probably why larvae treated with X34 avoided the mortality outbreak observed in untreated ones after 27 dpf. The improvement of survival associated with *Phaeobacter sp.* treatment had already been demonstrated in hatchery for *Ostrea edulis* (Prado 2006) and *P. maximus* (Ruiz-Ponte et al. 1999) and for *C. gigas* using bacterial challenges in small-scale bioassays (Kersarcodi-Watson et al. 2012; Karim et al. 2013). However, in Ruiz-Ponte, the efficient protection of scallop larvae exerted by *P. gallaeciensis* was demonstrated only with the bacterial cells broken down using an ultra-sonic treatment while no effects was observed with entire bacteria. These results contrast with those observed in our study where a probiotic effect was observed when entire X34 bacteria were added to larval cultures. This difference could be explained by the implementation of a ten days pre-treatment period before the bacterial challenge experiment. Indeed, Karim et al. (2013) showed that the probiotic ability of *Phaeobacter sp.* was significantly enhanced when larvae were exposed for 24 h before bacterial challenge. This suggests that X34 must colonize the larvae to efficiently protect them against further pathogen infection. The absence of a long pre-treatment period could also explain the lack of X34 protective effects against *V. pectenicida* observed by Kersarcodi-Watson et al. (2012) when bacterial challenges were performed on *P. maximus* larvae using small-scale bioassays. These findings suggest that X34 treatment can be beneficial in controlling the proliferation of pathogenic bacteria, but that it is not well suited as a curative method once high bacterial loads have set in. This is in accordance with what is expected from a probiotic, which in this case is to

pre-emptively favour the host's disease resistance. It remains to be verified if X34 treatment can be used to prevent mass mortality events frequently observed in hatcheries (especially for *P. maximus*) and which are associated with the proliferation of one or more bacterial pathogens in rearing tanks.

Beside the improvement in survival after bacterial challenge, the probiotic treatment lead to higher double ring rates at 29 dpf (25% higher) comparatively to untreated ones, suggesting better metamorphosis abilities for X34 treated larvae. However, at 29 dpf, the level recorded in X34 larvae was similar to those observed in the antibiotic treatment and in unchallenged larvae. This indicates that probiotic treatment did not enhance metamorphosis ability, but rather that probiotic treated larvae were in a better physiological condition to undergo more rapidly metamorphosis, particularly in the survival after *Vibrio* exposition. Part of the explanation can be found in the results from the spectrophotometric analyses. Indeed, we measured an activation of antioxidant defenses (CAT and SOD) 48 h after pathogen exposition in X34 treated and untreated larvae with significantly higher levels in untreated larvae, while no changes in lipids peroxidation (MDA) were recorded. After Genard et al. (2011, 2013), the activation of antioxidant defenses after a bacterial challenge could be associated to the immune response activation in order to manage the production of various reactive oxygen species (ROS) during the intracellular destruction of phagocytized bacterial material, thus avoiding the peroxidation of host tissues. In support of this, our results indicate that larvae developed an accurate immune response triggered by the bacterial infection. However, the authors also showed that immune response activation implies energy allocation which declines the energetic budget of larvae. Thus, the higher CAT and SOD activities measured in untreated larvae could indicate that energy invested in the immune response was higher comparatively to larvae treated with the probiotic. This could in turn provoke a weakening of the larvae and limit the energy available for larval development and/or further activation of accurate immune and antioxidant processes. Subsequently, the lower survival and metamorphosis rates measured in challenged untreated larvae could be related to the progressive weakening induced after the bacterial infection rather than as a direct effect of pathogen activity.

To conclude, in this study, after an in vitro validation of X34's probiotic abilities, we investigated its effectiveness for *P. maximus* cultures in the context of a bacterial challenge. Comparatively to bacterial challenges performed on small-scale bioassays (Kersarcodi-Watson et al. 2009, 2010, 2012; Karim et al. 2013), our approach simulated conditions more representative of those found in a commercial hatchery (in terms of larval density, hatchery routine and larval development). Thus, our results are complementary to these previous works and validate the usefulness of X34 for *P. maximus* larval production. However, while the effect against a pathogen attack was demonstrated, the real potential of X34 still needs to be evaluated at a commercial scale. In a similar line of tought, a simpler delivery system than the supply of fresh probiotic cultures should be assayed such as freeze-dried cultures or probiotics associated with algae as suggested by D'Alvise et al. (2012). Otherwise, our results highlight the importance of a pre-treatment period with the probiotic to ensure an accurate response to infection. It suggests that probiotics should be delivered early during development and the most appropriate moment (after fertilization, D-larvae or veliger stages) should be investigated in further experiments.

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