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Abstract

The European sea bass (*Dicentrarchus labrax*) is one of the most produced marine fish species in Europe. Its larval stage is particularly sensitive and is thus characterized by relatively low survival rates, making it acutely vulnerable to multiple infectious hazards that can occur during its intensive production cycle in hatcheries and at sea. In this study, we investigated the potential probiotic effect of marine *Pseudoalteromonas* bacterial strains against two major pathogens of this species, *Vibrio harveyi* (a bacterial pathogen) and the nervous necrosis virus (NNV); we also investigated antibiofilm effect of these *Pseudoalteromonas* strains. Over an 8 to 12-week impregnation phase, seabass juveniles were immersed fortnightly for 4 hours in static hyperoxygenated seawater containing the probiotic candidates of *Pseudoalteromonas* strains at a concentration of 10^6 CFU/mL. We tested four candidates: (1) a combination of two strains producing antimicrobial compounds, h*Cg*-42 and h*Oe*-125; (2) strain 3J6, with known antibiofilm properties and (3) strain RA15, from the same genus, but with no identified probiotic effect. At the end of the impregnation phase, fish underwent an infection challenge with an intraperitoneal injection at a dose of 4.6×10^8 CFU/mL of *V. harveyi* or a 4-hour bath with a pathogenic strain of NNV. Thereafter, mortality was monitored for 2 and 6 weeks, respectively, at temperatures allowing the development of clinical signs. Immunological analyses were carried out during impregnation and after infection. The probiotic candidates were detected at different sampling times after the 4-hour immersion session in the gills and mucus, but there was no evidence of long-term persistence. For the *V. harveyi* challenge, no statistical difference in mortality was observed between the non-impregnated control (63%) and the 3J6-impregnated (68.7%) group, but improved survival rates of 10 and 25% were obtained for the RA15- and the double strain (h*Cg*-42+h*Oe*-125)-impregnated groups, respectively. For the NNV challenge, no significant benefic effect of the probiotics on infection kinetics or cumulative mortality was observed. Leucocyte mortality and phagocytosis activity revealed only slight significant differences between the treatment groups, either during impregnation or after infection challenges. Regarding biofilm development during impregnation with probiotic candidates, the maximal thickness of biofilm was significantly lower in the 3J6, double-strain and RA15 groups, compared with the non-impregnated control group. This study highlights the interesting probiotic potential of marine bacteria to limit mortalities induced by bacterial pathogens as well as biofilm development. Further investigations are in progress to investigate the mechanisms of action of these probiotics and to improve their formulation for larger-scale tests.

Introduction

The European sea bass (*Dicentrarchus labrax)* is naturally found in the European waters, from the Northeast Atlantic (off Norway) to the Mediterranean Sea [1]. Sea bass aquaculture represents an important economic market with more than 191 000 tones (t) produced worldwide in 2016, out of which 82 000 t were farmed in Europe [2]. Even though zootechnical improvements have strongly contributed to the optimization of sea bass production over the last decades, the farmed sea bass market is threatened

by episodes of epidemics caused by pathogenic agents such as bacteria, viruses or parasites [1], which can result in substantial mortalities and economic losses [3].

Among bacterial diseases affecting sea bass, vibriosis includes infections caused by different Gram-negative *Vibrio* species such as *Vibrio alginolyticus* [4], *Vibrio parahaemoliticus* [5], *Vibrio anguillarum* [6–8] and *Vibrio harveyi* [7, 9]. Infection by *V. harveyi* was first reported to induce ulcerative lesions in European sea bass in the late 1990s [7]. The disease is characterized by lethargy, exophthalmus, ulcers and skin lesions, hemorrhaging in the internal organs, ascites and paleness of the kidney and liver. *V. harveyi* occurs naturally in marine habitats [10] and it has already been described as a pathogenic species for a large range of marine animals, from mollusks to crustaceans and fish [11]. This pathogen affects several fish species, causing a large range of symptoms [12] such as skin ulcerations in *Solea senegalensis* [13], tail rot disease in *Sparus aurata* [14] or necrotizing enteritis in *Paralichthys dentatus* [15].

During the past decades, several strategies to fight against *V. harveyi* infection have been developed to limit the use of antibiotics [12], including phage therapy [16], dietary supplements with probiotics [17] or inhibition of quorum sensing [18]. In 2019, a study investigated the protective efficacy of a *Pseudoalteromonas* strain in European abalone against infection by *V. harveyi* ORM4 [19]. That study demonstrated that abalone immersed prior to infection in a seawater suspension of *Pseudoalteromonas* h*Cg*-6 — isolated from the hemolymph of healthy marine mollusks [20] — showed significantly improved viability, with a 24% increase in the survival rate [19]. *Pseudoalteromonas*, well known to be associated with marine organisms, produce antimicrobial compounds such as alterins, which may contribute to host defense [21, 22]. Alterins are considered to be one of the largest families of antibacterial cyclolipopeptides from marine bacteria [22]. They are especially produced by *Pseudoalteromonas* strains h*Cg*-6, h*Cg*-42 and h*Oe*-125. Alterocin, a 139 amino acid protein is produced by *Pseudoalteromonas* sp. 3J6 and exhibits antibiofilm effects without bactericidal activities, which is rare and original [23].

Viral encephalopathy and retinopathy induced by the nervous necrosis virus (NNV), a betanodavirus, is one of the most important diseases for European sea bass. Described in the 1990s, it has been reported to cause mass mortalities in hatcheries and aquaculture farms, as well as in the natural environment [24–27]. No efficient treatment or vaccine exists to date to fight against this pathogen, which has an extremely wide host spectrum and significant recombination capacities due to its genome composed of two RNA fragments [28].

In this study, we investigated the potential of *Pseudoalteromona*s strains h*Cg*-42, h*Oe*-125 and 3J6 to improve the resistance capacities of European sea bass, after impregnation for several weeks with these probiotic candidates, against *V. harveyi* and NNV. After characterizing the efficiency of the immersion process used to impregnate the fish with the *Pseudoalteromonas* strains, sea bass were repeatedly immersed with the probiotic candidates over a period of at least 2 months, and then experimentally infected by V. *harveyi* or NNV. The presence of probiotic candidates in fish, their impact on biofilm

parameters, the kinetics of mortalities associated with the infections and various markers of the immune system were monitored during the impregnation and/or infection phases.

Materials And Methods

1. Production of the probiotic suspensions

The bacterial strains used in this study are described in Table 1. *Pseudoalteromonas* strains h*Cg*-42, h*Oe*-125 and 3J6 were selected as probiotic candidates. The *Pseudoalteromonas* strain RA15, which does not produce any alterin or alterocin compounds, was used as a control strain with no expected antibacterial effect. *Pseudoalteromonas* strains were grown on Marine Agar (Sigma-Aldrich) or in 5 mL Marine Broth (Sigma-Aldrich) with agitation at 150 rpm for 24 h. A volume of 2.5 mL of this pre-culture was then added to a final volume of 220 mL of Marine Broth, cultured for 24 h in the same conditions and centrifuged at 3220 × *g* for 25 min. After removing the culture medium, the cell pellet was washed twice, i.e. centrifuged at 3220 × *g* for 30 min, and re-suspended in sterile SeaSalts (Sigma-Aldrich) at 3% to obtain the final suspension for the immersion procedure. The final concentration of the suspension was controlled by performing serial dilutions in duplicate on Marine Agar, from 10^{-1} to 10^{-8}, for each strain tested. After two days of culture, the concentrations obtained were over 10^{9} colony-forming units (CFU)/mL.

Table 1
Bacterial strains used in this study

Strain	Description	Production of alterins ou alterocin	Reference
Pseudoalteromonas h*Cg*-6	Type strain with antibacterial activity isolated from hemolymph of *Crassostrea gigas*	Alterins	[20, 22, 47]
Pseudoalteromonas h*Cg*-42	Strain with antibacterial activity isolated from hemolymph of *Crassostrea gigas*	Alterins	[20, 22, 47]
Pseudoalteromonas h*Oe*-125	Strain with antibacterial activity isolated from hemolymph of *Oestrea edulis*	Alterins	[22, 48]
Pseudoalteromonas sp. 3J6	Strain with antibiofilm activity isolated from an underwater substratum	Alterocins	[23, 44, 49]
Pseudoalteromonas rhizospherae RA15	Strain isolated from *Arthrocnemum macrostachyum*, plant growth promoter, without any antibacterial or antibiofilm activity. Used as a negative control	None	[50]
Vibrio harveyi no. 94473 1811603 AQN553P2	Pathogenic strain provided by Dr. Alain Le Breton – VetEau	None	[7]

2. Maintenance of European sea bass (Dicentrarchus labrax)

Fish experimentation was carried out in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Directive 2010/63/EU). Experimental procedures were validated by the ANSES animal ethics committee (ANSES/ENVA/UPC No. 16) and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS #32741-2020121509556347 v5). Two batches of European bass from the hatcheries at the Fermes Marine du Soleil (https://fermesmarinesdusoleil.com) fish farm were used in this study. Batch 1 was composed of 1500 juvenile sea bass with an average weight of 15 g. They were vaccinated against *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida*; this vaccination does not confer any cross-protection against *V. harveyi* infection. Batch 2 was composed of 1500 juvenile sea bass with an average weight of 5 g, not vaccinated to avoid interference with the NNV challenge. All batches were free of viral septicemia hemorrhagic virus (VSHV), infectious hematopoietic necrosis virus (IPNV), infectious pancreatic infectious virus (IHNV) and NNV. Their health status was checked during the quarantine period following their arrival, with autopsies and general bacteriological and virological analyses.

Fish were kept in filtered seawater (treated on sand and activated carbon filters), sterilized with UV. The water circulation was continuous (with tank water renewal occurring at least once hourly), with a temperature regulated at 20°C ± 2°C and an oxygen saturation greater than or equal to 80%. Throughout the rearing protocols, the fish received a diet appropriate for their weight and adapted to the water temperature (Le Gouessant, pellet diameter ranging from 1.7 to 3.2 mm). If needed, compassionate euthanasia was employed in the event of observation of signs of suffering or at the end of the experiment. Euthanasia involved the addition of a lethal dose of 100 ppm of Eugenol (Fili@Vet Reseau Cristal) to the tank water. Fish were exposed to Eugenol until the complete disappearance of all respiratory activity ceased.

3. Impregnation of sea bass with Pseudoalteromonas

3.1. General procedure

The impregnation procedure consisted in immersing the fish for 4 h in a bath of limited volume of hyperoxygenated seawater containing the probiotic strains at a determined concentration. At the end of this session, the tanks were again continuously supplied with seawater. Fish were observed at least every 30 minutes during immersion to visually check for abnormal behavior.

3.2. Assessment of the safety of the Pseudoalteromonas strains for European bass

The safety of the tested *Pseudoalteromonas* strains for the European bass was assessed by immersing three fish from Batch 2 in 5 L of hyperoxygenated seawater at 20°C ± 2 for 4 h at a concentration of 10^7 CFU/mL of *Pseudoalteromonas* strains, which is tenfold higher than the concentration selected for the impregnation procedure. Experimental treatment groups were composed of *Pseudoalteromonas* strain h*Cg*-42 combined with strain h*Oe*-125, *Pseudoalteromonas* 3J6, *Pseudoalteromonas* RA15 and a control

with no bacteria added. The overall condition of the fish (behavior, mobility and respiration) was followed for 1 week after this impregnation.

3.3. Evaluation of the persistence of probiotic candidates in fish after one immersion session

Experimental immersions (see Section 3.1.) were performed using 35 fish from Batch 1 per tank in 8 L of seawater following three impregnation treatments: (1) with strain 3J6, in duplicate; (2) with a combination of strains h*Cg*-42 and h*Oe*-125 in duplicate and (3) without bacteria (control). The final concentration of the probiotic candidates was 10^6 CFU/mL of seawater for each. Right before and after this immersion session, fish were transferred to 50 L tanks, and mucus and gills were sampled on three fish per impregnation treatment to detect probiotic candidates (T0 and T4H). This sampling procedure was repeated at 24 h, 48 h, 72 h and 96 h post-immersion (n = 3 fish per time point and impregnation treatment). Tissues were quick-frozen in liquid nitrogen and then conserved at -80°C until analysis. This procedure is described in Fig. 1.

3.4. Long-term impregnation

The experimental procedure is described in Fig. 2. Four experimental treatment groups were formed: (1) Control, (2) strain RA15, (3) combined strains h*Cg*-42 + h*Oe*-125 and (4) strain 3J6. For Batches 1 and 2, each group was composed of four biological replicates of 50 L tanks containing 70 sea bass per tank, except for the control group that included five biological replicates. Immersion was carried out in a volume of 12 L of seawater containing 10^6 CFU/mL of each tested strain. This protocol was repeated every two weeks for 8 weeks for Batch 1 (total of four immersion sessions) and 12 weeks for Batch 2 (total of six immersion sessions). For the control treatment, no bacteria were added to the tanks. Samples of mucus and gills were taken twice, after 4 h of immersion, to detect the probiotic candidates. This sampling was done at T0 (i.e. after the first immersion session) and T2 (after the second immersion session, 2 weeks after the beginning of the experiment) for Batch 1 and at T0 and T4 (4 weeks after the beginning of the experiment) for Batch 2.

4. Analysis of biofilm formation

Biofilm formation was monitored on 76 × 26 mm glass slides during the impregnation of fish from Batch 2. To do so, slides were immersed in fish tanks during the first 8 weeks of impregnation. Thereafter, the slides were collected and stored at + 4°C for 24 h in tank seawater. For analyses, slides were rinsed twice in sterile seawater, dried and stained with 15 µL of 5 µM SYTO-9 (Invitrogen – Life Science Technology) for 45 min. SYTO-9 fluorescence was measured with an 488 nm excitation filter and a 500 and 550 nm emission filter by confocal laser scanning microscopy, CLSM, (Zeiss, LSM710) using a 63x oil immersion objective. At least three images were acquired for each slide. 3D images were acquired using ZEN software (Zeiss). ZenBlue software (Zeiss, license-free version) was used to process data from the 3D images. The biofilm stacks were analyzed using COMSTAT software [29] to estimate the biovolume ($\mu m^3/\mu m^2$),the average thickness (µm) and the maximum thickness (µm).

5. Effects of the Pseudoalteromonas strains against V. harveyi and NNV

5.1. Selection of the fish pathogen isolates

The infectious strain of *V. harveyi* no. 94473 1811603 AQN553P2 used in this study, kindly provided by Dr. Alain Le Breton (Vet'Eau), was isolated from sea bass during an episode of mortality (Table 1). This strain was cultivated on Tryptic Soy Agar (TSA) medium at 25°C. The red-spotted grouper NNV strain PP160 was produced and titrated on a snakehead fish cell line (SSN-1 cells), as previously described [30]. The viral suspension was kept at -80°C until used.

5.2. In vitro characterization of the effect of Pseudoalteromonas strains against V. harveyi

The antimicrobial activity of *Pseudoalteromonas* strains against *V. harveyi* was assessed using the well-diffusion method. Briefly, bacterial strains were grown in Marine Broth at 18°C under shaking (100 rpm) for 24 h for *V. harveyi* or 72 h for *Pseudoalteromonas* strains (*Pseudoalteromonas* h*Cg*-42, *Pseudoalteromonas* h*Oe*-125, *Pseudoalteromonas* 3J6 as a positive control strain and *Pseudoalteromonas* RA15 as a negative control strain). Then, 1 mL of each *Pseudoalteromonas* culture was centrifuged (6000 × *g* for 5 min at 4°C) and the supernatant was inactivated 5 min at 100°C before being tested against target bacteria. To test the activity of these supernatants, Marine Agar plates were inoculated on the surface with 5 mL of an exponential growth culture of *V. harveyi* adjusted to 1×10^6 CFU mL^{-1}. When dry, wells (4 mm in diameter) were punched into the Marine Agar medium and supernatants (20 µL) or controls (Marine Broth for negative control and polymyxin B at 1 µg mL^{-1} as positive control against Gram-negative target bacteria) were dropped into agar punch holes of 0.4 cm. After overnight incubation at the optimal growth temperature of the strain, antibacterial activity was detected based on the appearance of an inhibition halo around wells.

5.3. Experimental infection of impregnated sea bass with V. harveyi

After two steps of cultivation on TSA medium at 25°C for 24 h, 5 mL of TSB was added to the culture plate to resuspend the bacteria. Optical density (OD) was measured at 600 nm wavelength and the suspension was diluted in TSB to calibrate the concentration for fish injection.

To define the 50% lethal dose (LD50), four different concentrations of *V. harveyi* were tested in 10 fish per concentration (from Batch 1), by intraperitoneal injection (IP) (100 µL per fish). The LD_{50} was defined as the concentration inducing the mortality closest to 50% one week after infection at a water temperature of 20°C ± 2. For the infection, European sea bass resulting from long-term impregnation (50 fish from Batch 1 with an average weight of 32.6 g per tank; 4 replicates per treatment), were anesthetized with Eugenol (1:500 final concentration) and individually IP injected with a volume of 100 µL containing 4.6×10^8 CFU of *V. harveyi* (Fig. 2). One tank of the control group was treated with sterile TSB as a negative control of the infection (i.e. non-infected control group). Mortalities were followed daily for 2 weeks at 20°C ± 2 and all survivors were euthanized with Eugenol.

5.4. Experimental infection with NNV

Following Castric et al. [31], a stock of betanodavirus, PP160 strain (red-spotted grouper nervous necrosis virus [RGNNV/RGNNV] genotype), isolated from diseased *D. labrax* displaying typical signs, was produced at 24°C on the SSN-1 cell line (L15 medium, 10% FBS, pH7.6) and frozen 10 days post-inoculation. Cell debris were removed by centrifugation for 15 min at 2000 × *g*, the virus was then aliquoted and stored at − 80°C. Before the challenge, viral titration was carried out on one of the aliquots after a single freeze-thaw cycle based on the tissue culture infectious dose technique (TCID50) described in Castric et al. [31]. The infectious titer of the viral production was calculated according to the Kärber method [32], and was found to be 1×10^8 TCID50/mL. Fish from Batch 2 (17 tanks with approximately 54 fish per tank) were immersed for 4 h in a static bath of 6 L of aerated seawater at 26°C ±2 containing 1×10^5 TCID50/mL of the PP160 strain. Negative control fish (with no probiotic supplementation) underwent the same protocol, but using non-infected SSN-1 cell supernatant. Mortalities were followed daily during 6 weeks at 26°C ± 2 and all survivors were euthanized with Eugenol.

6. DNA extraction procedure

DNA of a 2 mL pure culture of each *Pseudoalteromonas* strain was extracted using the Monarch Genomic DNA Purification Kit (Biolabs, protocol without lysozyme); and DNA of mucus and gills was extracted using the DNeasy PowerSoil Pro Kit (Qiagen). For gills, 250 mg was placed in 2 mL Lysing Matrix D tubes (MPbio) containing 1.4 mm ceramic beads. Sterile PBS was added to obtain a volume-to-weight ratio of 1.6:1 and tissues were homogenized using the Precellys Evolution tissue homogenizer equipped with the Cryolys Evolution cooling system, at 4°C and 10 000 rpm, 4 × 30 s. After homogenization, 400 µL was used for DNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen) homogenization tubes with 800 µL of solution CD1 and again homogenized at 4°C and 4500 rpm, 2x 30 s. Mucus extraction was initiated directly at this stage. After this step, DNA was extracted according to the manufacturer's recommendations for both gills and mucus. DNA was eluted in 100 µL of elution buffer and concentration was measured using the NanoDrop 1000 spectrometer (ThermoScientific). DNA was diluted to 5 ng/µL for use in qPCR analyses.

7. qPCR analyses for quantification of Pseudoalteromonas strains

qPCRs were performed using the CFX Touch96 thermocycler (Bio-Rad) and the qPCR kit Fast start essential DNA green master (Roche LifeScience). The primers OB-PVP 3 (5'-CTTGCTTGGAAATGGGCTGA-3') and OB-PVP 4 (5'-TGGCCCGTAAGCATTGTATAAA-*3'*) were designed to target a 51 bp CDS, specific to *Pseudoalteromonas* strains h*Cg*-6 (CDS HCG6B_v1_a1650, accession number CP102371-CP102372), h*Cg*-42 (CDS HCG42B_v1_3844, accession number CP118496-CP118498) or h*Oe*-125 (CDS HOE125B_v1_a0382, accession number CP118914-CP118915), without discrimination (same CDS sequence with different names). As these CDSs are common to the *Pseudoalteromonas* antimicrobial strains (hCg or hOe), this sequence will be called AntiMicCDS in the manuscript. Used on a mix of two strains, this qPCR did not allow discrimination, but gave a copy number of the *AntiMicCDS* as a proxy of the quantity of the two strains. The primers OB-PVP 5 (5'-CTTTCAGCAAACACAATGGCA-3') and OB-PVP 6 (5'-GCCTTGTCGCTCTTCCACAT-3') were designed to target the gene coding for the alterocin that allow

detection of the *Pseudoalteromonas* strain 3J6. Each qPCR reaction (20 µL final volume) was composed of 2 µL of a mix of primers 3 + 4 or 5 + 6, diluted to 3.75 µM in sterile milliQ water, 10 µL of the SYBR Green qPCR mix solution and 8 µL of DNA diluted at 5 ng/µL.

Cycling conditions were as follows: 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 10 s. A melting curve analysis was performed at the end of the qPCR at 95°C for 15 s, 60°C for 10s, and 95°C for 15 s. Each run included a blank control (H_2O control) and appropriate positive DNA controls used to perform a standard curve from 10^5 copies/µl to 10^1 copies/µl. The LD_{PCR} (limit of detection of the PCR) was determined according to the standard NF U47-600-2. Briefly, dilutions of bacterial DNA were tested three times in qPCR in 8 replicates for each strain. LD_{PCR} was defined as the last number of copies at which amplification (level defined as the threshold cycle, Ct) can be determined on at least 23 of the 24 replicates of a dilution. In this study, the LD_{PCR} corresponded to 5 copies for both *AntiMicCDS* and *alterocin* gene amplification. The qPCR results were analyzed using CFX MAESTRO software (Bio-Rad).

8. Immune system analyses

Immune parameters were monitored based on flow cytometry analyses using a protocol adapted from Danion et al. [33]. For each fish sampled, an approximate volume of 300 µL of blood was withdrawn from the caudal vein using a lithium heparin vacutainer (BD Vacutainer LH 85 U.I.). These whole blood samples were diluted with 7 mL of Leibovitz 15 medium (L15 Eurobio) containing 20 U heparin lithium (Sigma). Then, an aliquot of 7 mL was loaded on a Ficoll gradient (Histopaque® 1077, Eurobio) to a density of 1.077 g cm^{-3}. After centrifugation (400 × *g*, 30 min, 15°C), leucocytes at the interface were collected and washed twice (1200 × *g*, 5 min, 4°C) with L15 medium. Phagocytosis activity and leucocyte mortality were analyzed on an Attune NxT Acoustic Focusing Flow Cytometer (Invitrogen Molecular Probes). For each sample, 15,000 events were counted and subpopulations (i.e. lymphocytes, monocytes and granulocytes) were subsequently determined using gates based on size and complexity in the general acquisition data. Phagocytosis activity was analyzed on 300 µL of leucocyte samples and 15 µL of a 1:10 dilution of fluorescence beads (2.7×10^{10} particles mL^{-1}, Fluorosphere® carboxylate-modified microspheres, diameter 1 µm, MolecularProbes) after incubation for 30 min at room temperature. Using green fluorescence, phagocytosis activity was determined on the monocyte/granulocyte population. Fluorescence beads were first analyzed alone to remove the area that corresponds only to these beads, not to the mix of beads and leucocytes.

Cell mortality was analyzed on 300 µL of leucocyte suspensions after 30 min incubation at 4°C in the dark with 15 µL of propidium iodide (PI, 1.0 g L^{-1}, MolecularProbes). This procedure was carried out on whole immune cells without distinguishing subpopulations during all data acquisition. Dead cells were counted in the red fluorescence gate, because only dead cells are able to internalize the red fluorescent PI.

For Batch 1 (*V. harveyi* challenge), immune parameters were analyzed at 15 days post-infection (dpi) (i.e. at the end of the infection challenge) on 6 fish per treatment (n = 4 for the negative control). For Batch 2 (NNV challenge), immune parameters were analyzed at the end of the impregnation phase (12 weeks of

impregnation) on 8 fish per treatment and during the infection phase at 96 hours and 7 dpi on 6 fish per treatment per sampling time.

9. Statistical analyses

All statistical analyses were performed using multiscale comparison tests. Specifically, we performed an ANOVA test followed by the Tukey HSD post-hoc analysis or a Kruskal-Wallis test followed by the Dunn test for post-hoc analysis depending on our data.

Results

1. Safety of *Pseudoalteromonas* strains for sea bass

Immersion of European bass during 4 h with 10^7 CFU/mL of probiotic candidates, a quantity 10 times greater than that used subsequently, did not induce any sign of animal suffering or any behavior change in the week following immersion. No mortality was observed.

2. Persistence of the probiotic candidates in fish tissues after one immersion session

In the gills, an average of 1.20×10^4 copies of the *AntiMicCDS* and *alterocin* genes were detected at the end of the immersion step (T4H) but no persistence was observed after this time (T24H, T48H, T72H and T96H post-immersion; Fig. 3).

In the mucus, the *AntiMicCDS* and *alterocin* genes were detected at 4.95×10^4 and 2.49×10^4 copies on average, respectively, after the 4 h immersion session (Fig. 4A). Significant levels of the *alterocin* gene were still found after 3 to 4 days post-immersion (Fig. 4B). Results of the qPCR on the control fish showed that some marine bacteria present naturally on the fish carried the *alterocin* gene, but at a level much lower than that detected in impregnated fish.

3. Detection of the *Pseudoalteromonas* strains during long-term impregnation

Throughout the long-term impregnation, the average copy number of the *AntiMicCDS* gene was estimated at 9.3×10^3 in the gills and 4.8×10^5 in the mucus of the fish from Batch 1, and at 2.13×10^3 in the gills and 1.44×10^5 in the mucus of the fish from Batch 2. For the 3J6 group, the average number of *alterocin* gene copies was estimated at 5×10^3 in the gills and 3.5×10^5 in the mucus for the fish from Batch 1 and at 3.3×10^3 in the gills and 4.61×10^4 in the mucus for the fish from Batch 2.

4. Biofilm development during impregnation with *Pseudoalteromonas* strains

Mean thickness (µm), biovolume ($\mu m^3/\mu m^2$) and maximal thickness (µm) of the biofilms that formed on the surface of glass slides immersed 8 weeks in water with impregnated or control fish are shown in Figure 6A, 6B and 6C, respectively. For these three parameters, the control slides were always statistically different from those immersed with at least one of the other experimental groups. The maximal thickness

of the biofilm on the control slides (16.47 µm) was significantly higher than those immersed with the experimental groups (10.22µm; 11.77µm; 11.62µm for RA15, Combined strain and 3J6 groups respectively). Also, the biovolume of the biofilm on the control slides was 10.54 $\mu m^3/\mu m^2$ in average against 6.60 $\mu m^3/\mu m^2$ in RA15 group; 8.83 $\mu m^3/\mu m^2$ in the combined strains group; 9.84 $\mu m^3/\mu m^2$ in 3J6 group. As we can observe in Figure 6E to 6G, the biofilm was also composed of different type of organisms, such as microalgae and bacteria and was not homogenously distributed on the slide. Indeed, a reduction of maximal thickness and of biovolume in the impregnated-groups suggest modification of biofilm architecture in these experimental groups.

5. Impact of the probiotics on *V. harveyi*

5.1. In vitro effects

The culture supernatant of *Pseudoalteromonas* h*Cg*-42 inhibited *V. harveyi* growth. However, the culture supernatant of *Pseudoalteromonas* h*Oe*-125, 3J6 and RA15 did not show antibacterial activity against the *V. harveyi strain* used in this study (Fig. S1).

5.2. Monitoring of cumulative mortalities during the *V. harveyi* challenge

Cumulative mortalities after IP injection of *V. harveyi* are presented for each group in Figure 7. The percentages of survival within a group were first compared using an ANOVA test to analyze the inter-tank variability. One replicate from the infected-control group and one replicate from the h*Cg*-42+h*Oe*-125 group differed statistically from the three other tanks (infected-control group: p values ranged from < 0.05 to < 0.001; h*Cg*-42+h*Oe*-125 group: p values ranged from 10^{-3} to 10^{-5}) and were removed from all further analyses. The percentage of survival in the non-infected control group was 100% after IP injection with sterile TSB medium. In the infected control and 3J6 groups, the survival rates were similar, with 37% and 31.3%, respectively (Kruskal-Wallis test, $p = 1$). In the h*Cg*-42+h*Oe*-125 group, survival was 61.5% and was statistically different from the RA15 group (*p-value*: 9.71×10^{-11}). Further, no statistical difference was observed between the h*Cg*-42+h*Oe*-125 and the non-infected control groups ($p = 0.18$). For the negative probiotic control strain RA15, the percentage of survival, 48.6%, was significantly different from the infected control group ($p = 4.96 \times 10^{-4}$).

6. Impact of probiotics on mortality induced by NNV infection

Cumulative mortalities after NNV infection are presented for each group in Figure 8. The percentage of survival in the negative control group was 88%. In the infected control, the h*Cg*-42+h*Oe*-125 and the RA15 groups, the survival rates were 54.72%, 56.44% and 55.01%, respectively. No statistical differences were observed among these three treatment groups, which were all statistically different from the negative control. For the 3J6 group, survival was 45.19%, statistically lower than all the other probiotic-supplemented groups and to the infected-control group.

7. Monitoring of the immune system parameters

7.1. Immune parameters at 15 days post-infection with *V. harveyi*

Flow cytometry analyses at 15 dpi showed that the percentage of dead cells only differed for lymphocyte populations (Figure S2). The group impregnated with strain 3J6 had a statistically lower percentage of dead lymphocytes compared with the infected-control group and the h*Cg*-42+h*Oe*-125 group (p-value <0.05).

Phagocytosis was measured on both the monocyte and the granulocyte populations (Fig. S3). In the group impregnated with strain 3J6, phagocytosis was lower than in the non-infected control (p = 0.009). No other statistical difference was observed for phagocytosis between the different groups.

7.2. Immune parameters before and after infection with NNV

Flow cytometry analyses were done at 12 weeks of impregnation (i.e. just before the infection), at 96 h post-infection and at 7 dpi (Figs. S4-S6). The percentage of dead cells only differed for the lymphocyte population at 7 dpi (Fig. S4). The group impregnated with strain 3J6 had a statistically greater percentage of dead lymphocytes compared with the infected control group and non-infected control group (p < 0.05).

Phagocytosis was measured on monocyte and granulocyte populations (Figure S6). In the h*Cg*-42+h*Oe*-125-impregnated group, a decreased phagocytosis rate was observed compared with the infected control (p < 0.05) at 7 dpi. No other statistical difference in phagocytosis was observed between groups.

Discussion

Among the large diversity of pathogenic *Vibrio* species found in the aquatic environment, *V. harveyi* is currently the most problematic species for European bass, repeatedly responsible for significant economic losses in intensive farms [7][34]. The main treatments used to limit the losses associated with vibriosis include antibiotic therapies and vaccination, but they have limited efficacy and can lead to antibiotic resistance [7, 12, 35, 36]. Over the past years, probiotics have emerged as alternatives to limit the spread of antibiotic resistance and promote the prevention of these opportunistic diseases [37]. Several feed suppliers now supplement their fish feed with probiotics (mostly lactic acid-producing bacteria or *Bacillus*). However, these probiotics have often been developed for terrestrial animals and are therefore not necessarily suitable for fish, which live in a different environment and have different physiological needs [38]. In our study, we focused on the genus *Pseudoalteromonas*, which was first described in 1995 [39]. *Pseudoalteromonas* are marine bacteria found worldwide. This genus contains 41 species and over 3772 *Pseudoalteromonas* strains have been isolated to date [21]. These species and strains are of ecological significance due to the production of natural anti-fouling substances [40, 41], as well as metabolites that possess antimicrobial or antibiofilm activity [21, 42–45]. This antimicrobial or antibiofilm potential was investigated in this study for fish application by testing the ability of three potentially probiotic *Pseudoalteromonas* strains to limit or even prevent mortalities induced by a pathogenic strain of *V. harveyi* in bacteria-impregnated European sea bass.

Although the oral route, administered through feed, is generally the most frequently used route to supplement animals with probiotics, we chose to deliver our probiotic candidates to the fish using repeated sessions of bath immersion, each lasting several hours. One possible field application of this administration route is a permanent drip of a stabilized probiotic solution of probiotics in fish farm tanks. We used a bath impregnation method to promote this impregnation through the fish environment, so as to limit the variability that can be associated with individual feed intake. We studied the fate of our probiotic strains in two major fish tissues in direct contact with the aquatic environment: the gills and the mucus. We found significant quantities of the probiotic strains in these tissues in the hours following immersion, but were not able to measure persistence over time. Screening for probiotics is frequent in assays of feed supplementation, particularly in the intestine [46], where probiotics administered via feed coatings can be more easily detected. The lack of apparent persistence of our probiotics in the tissues analyzed is not necessarily problematic if the impregnation process nevertheless has beneficial effects. We also investigated biofilm formation and structure during impregnation. Our results indicate that the maximal biofilm thickness in the non-impregnated control was significantly higher than in the repeated impregnation with probiotic candidates. Furthermore, considering the other two parameters of biofilm development (maximum thickness and biovolume), the non-impregnated control always differed from at least one other experimental treatment group. This difference suggests that the impregnation of sea bass with probiotic candidates tends to modify these biofilm parameters after 8 weeks of repeated immersion sessions. For the probiotic h*Cg*-42 + h*Oe*-125 and 3J6 strains, these results may be partly associated with the antimicrobial or antibiofilm properties of alterins or alterocin, respectively, although for the 3J6 group, there was no major reduction in the biofilm parameters measured after 8 weeks in seawater. For the RA15 strain, for which no antibiofilm or antimicrobial activity has been described to date, the biofilm also appeared to have been modified compared with the non-impregnated control, potentially highlighting an interesting property of this strain. However, we must note that the evolution of these parameters does not allow us to obtain information on the composition of the biofilm, especially in terms of pathogens.

The main result of this work is the drastic reduction in the cumulative mortalities in fish experimentally infected with *V. harveyi* impregnated with the h*Cg*-42 + h*Oe*-125 strains (25% survival gain) or the RA15 strain (10% survival gain). Because the fish were IP injected, it appears more likely that the *Pseudoalteromonas* strains led to an inherent improvement in their ability to fight the infection rather than an enhancement of the skin barrier through an antibacterial action. Although qPCR did not persistently detect the *Pseudoalteromonas* strains in the gills or mucus on the days following immersion, the metabolite compounds potentially produced by the strains, which had colonized the fish and/or which were present in their environment, seem to have been significantly effective in helping fight the infection. Indeed, strains h*Cg*-42 and h*Oe*-125 have been reported to produce alterins, which are cyclolipopeptides with demonstrated antimicrobial activity against several pathogens [22]. An improvement in survival rates had already been reported for the European abalone *Haliotis tuberculata* impregnated 4 h with the *Pseudoalteromonas* strain h*Cg*-6 and then injected with *V. harveyi* ORM4 [19]. In this case, abalone were exposed to the probiotic strain right before the infection. Here, we demonstrated that the repeated

administration of probiotics had a significant effect on the survival rates after an infection challenge, which can occur several days after the last immersion bath.

Regarding the NNV challenge, we did not show any increase in the survival rate after impregnation with probiotic candidates. This result is not surprising because the candidates have been reported to influence bacterial development, not viral multiplication. This result suggests that repeated probiotic impregnation does not improve the capacity of sea bass to fight viral infections.

In the particular case of strain 3J6, the mortalities occurred more quickly than in the other treatments, especially in the non-impregnated and infected control treatment groups, and this strain did not appear to confer any protective effect. The mortalities reported in the viral infection were even significantly higher than in other conditions. Because this strain has a potential to limit bacterial biofilm formation, it may nevertheless be worthwhile to determine if the bacterial composition of the tank water (particularly in biofilms), may have increased the mortality rate by modifying the potentially protective microbiota and/or by inducing stress to the fish.

A surprising result in the case of *V. harveyi in*fection was the increase in the survival rate obtained with strain RA15, which has never been reported to produce any antibiofilm or antibacterial compounds. In the case of NNV infection, the results reported suggest that this strain showed the same pattern as in the combined h*Cg*-42 + h*Oe*-125 treatment. In our study, it appears that (i) the RA15 strain was able to produce one or more compounds different from the ones reported for strains h*Cg*-42, h*Oe*-125 and 3J6 with an impact on *V. harveyi* or (ii) RA15 may have outcompeted *V. harveyi*, thereby limiting its spread. This result highlights the importance of including this strain in future investigations.

Due to the very sudden nature of the death caused by *V. harveyi* on sea bass, it was not possible to harvest moribund animals; we therefore analyzed live fish at a defined time post-infection. At 15 dpi, flow cytometry analyses did not show differences among conditions, although the 3J6 strain showed a significant reduction in phagocytosis activity compared with the non-infected control group. Furthermore, during the viral challenge, we also observed slight modifications in immune parameters, without a clear, defined pattern. Further investigations are in progress to determine the effect of the impregnation on the microbiome of different sea bass tissues before and after a pathogen infection, but also to study other immune parameters such as gene expression or specific antibody response.

Finally, our results suggest that a higher dose of *Pseudoalteromonas* strains could further improve the survival rates after a bacterial infection. In the h*Cg*-42 + h*Oe*-125-impregnated fish, we doubled the survival rates compared with the RA15-impregnated fish. However, the number of bacteria in the dual strain impregnation was double that of the RA15 impregnation, because each strain was added at a concentration of 10^6 CFU/mL during the immersion sessions. Higher impregnation doses should therefore be tested, particularly given that a concentration of 10^7 CFU/mL did not reveal any toxicity for sea bass. Testing higher doses can help design an industrialization process, because the probiotics must

be produced at higher levels. Research efforts are also needed to investigate if the protective effect observed can be reproduced in real farm conditions and with bacterial pathogens other than *Vibrio*.

Conclusion

This study highlighted the protective effect of *Pseudoalteromonas* strains (h*Cg*-42 combined with h*Oe*-125 and strain RA15), significantly improving the survival rates of sea bass after experimental infection with *V. harveyi*. Based on a worldwide production of sea bass estimated at approximately 200 000 t in 2016, a 10 to 25% gain in survival following a bacterial infectious episode is economically significant for marine fish farmers. This improved survival could be further enhanced by a positive effect of these strains on the survival and the growth of the larval stages as well as on potential biofilm control. This proof of concept is a first step in the development of a solution applicable to sea bass farms, based on the potential of these marine bacteria in a circular aquaculture approach.

Declarations

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ETHICS COMMITTEE APPROVAL

All animal experiments were approved by the French Ministry of National Education, Higher Education and Research under the authorization number APAFIS #32741-2020121509556347 v5.

AUTHOR CONTRIBUTIONS

TM, YFl and MD acquired funds and coordinated the study. AR performed all experimental assays, the production of protective strains and the analyses of the samples harvested with advice of TM, MD, MB, YFr, ED and LP and LB. LP and YFl performed *in vitro* inhibition tests on *V. harveyi.* HC performed primers design for qPCR. AB, SR and AR performed biofilm measurement by microscopy. JC and LL produced the viral suspension for the NNV challenge. AR, TM and MD wrote the manuscript (first draft was written by AR). The manuscript was carefully reviewed by other co-authors, who all approved the final version.

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Figures

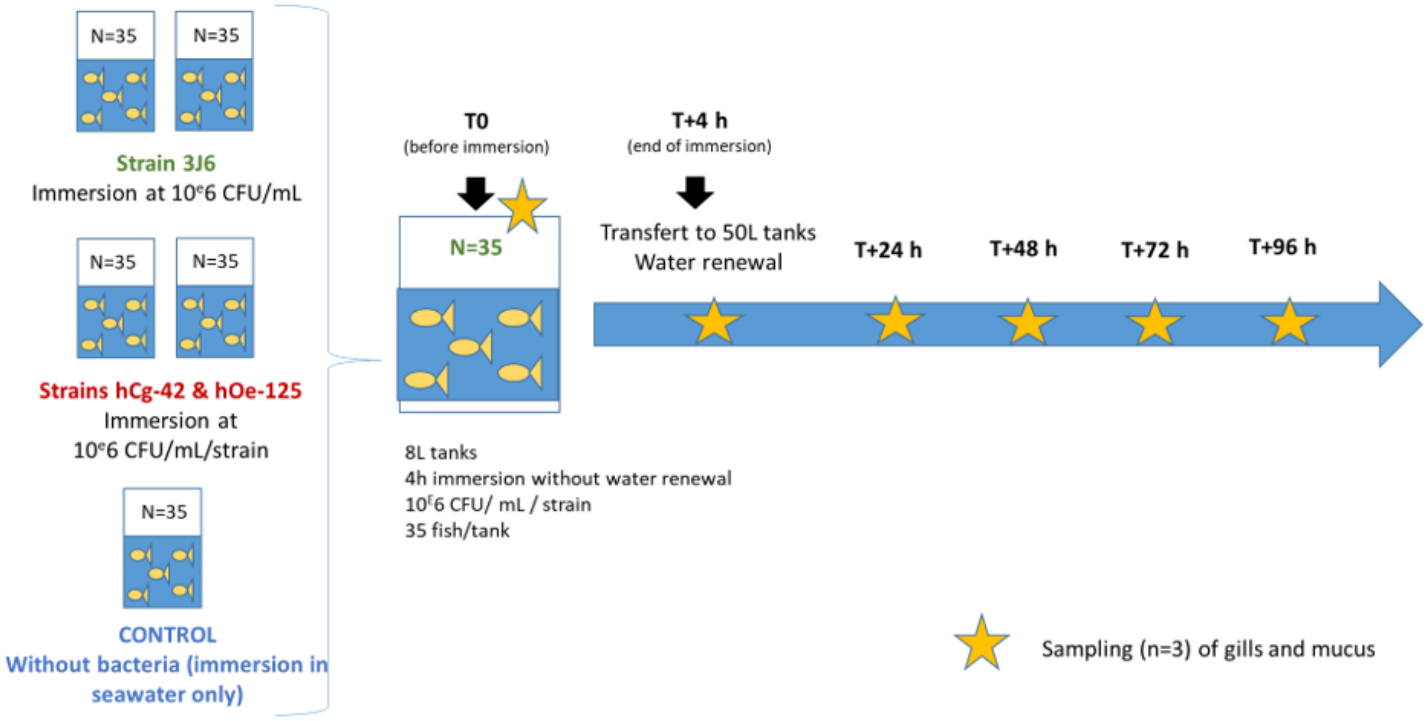

Figure 1

Assessment of the persistence of *Pseudoalteromonas* strains 3J6 and h*Cg*-42+h*Oe*-125 during the 96 hours following the immersion procedure. Persistence was tested on two biological replicates of 35 fish except for the control group (1 replicate of 35 fish). For the control, fish were immersed in seawater without supplementation with candidate probiotic bacteria. Stars represent the tissue samples (gills, mucus) taken on three fish in each tank and at different time points

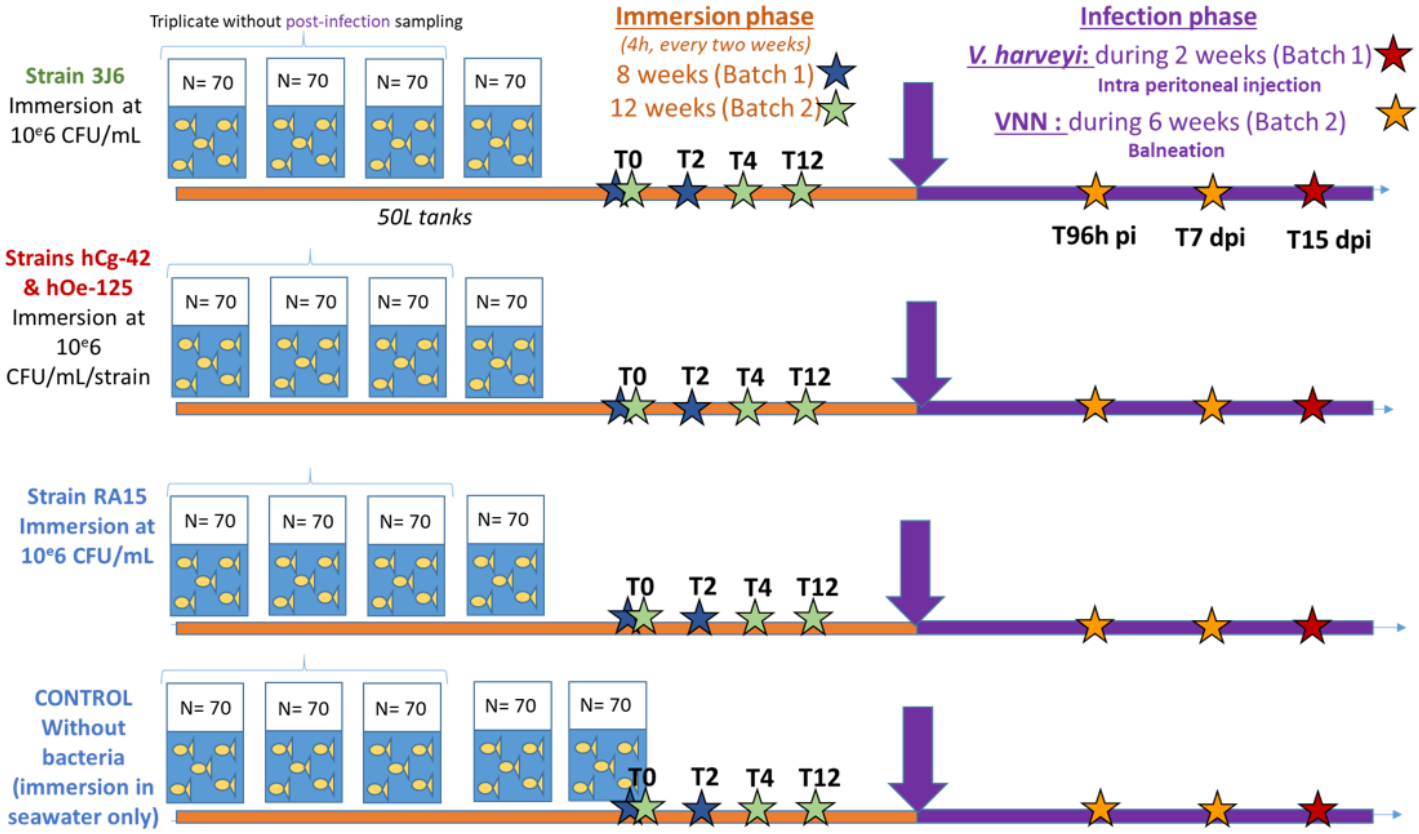

Figure 2

Experimental design of the long-term impregnation of European sea bass with *Pseudoalteromona*s strains and subsequent infection with *Vibrio harveyi* (Batch 1) or nervous necrosis virus (NNV) (Batch 2). A total of four experimental groups were formed: Control (without impregnation), RA15 (immersion with *Pseudoalteromonas*strain RA15), a combined strain group (immersion with *Pseudoalteromonas* strains h*Cg*-42 and h*Oe*-125) and 3J6 (immersion with *Pseudoalteromonas*strain 3J6). Stars indicate sampling times. Blue stars indicate sampling after a 4 h of immersion with the bacterial strains for Batch 1 and green stars for Batch 2. Red stars indicate the sampling after infection with *Vibrio harveyi* (Batch 1) and yellow stars indicate the sampling after infection with NNV (Batch 2). The number of fish is indicated for each batch

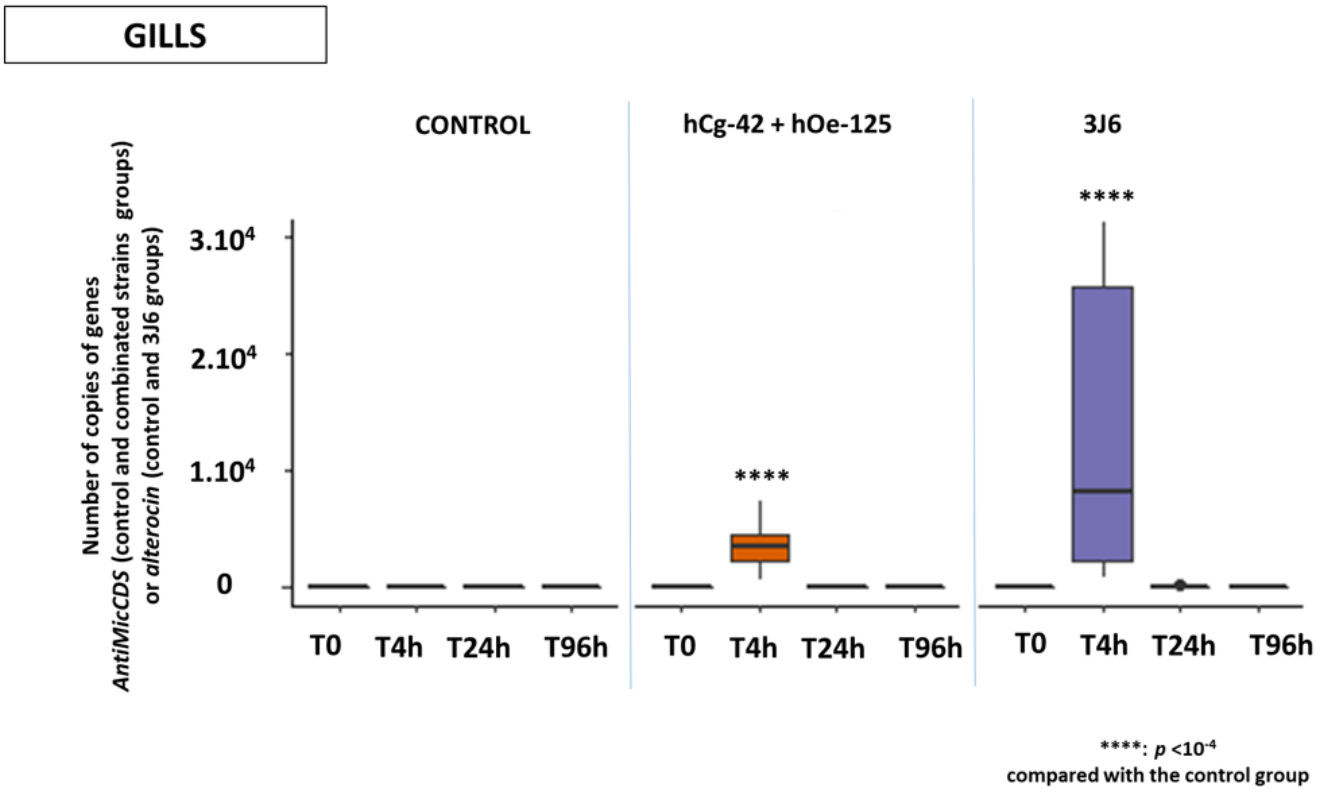

Figure 3

Copy numbers of the *AntiMicCDS (*representing strains h*Cg*-42 and h*Oe*-125) or *alterocin* (representing strain 3J6) genes in the gills at T0 (before the immersion), after 4 h of immersion (T4H), at 24 h and 96 h post-immersion for the control and probiotic treatments.**** : $p \leq 10^{-4}$

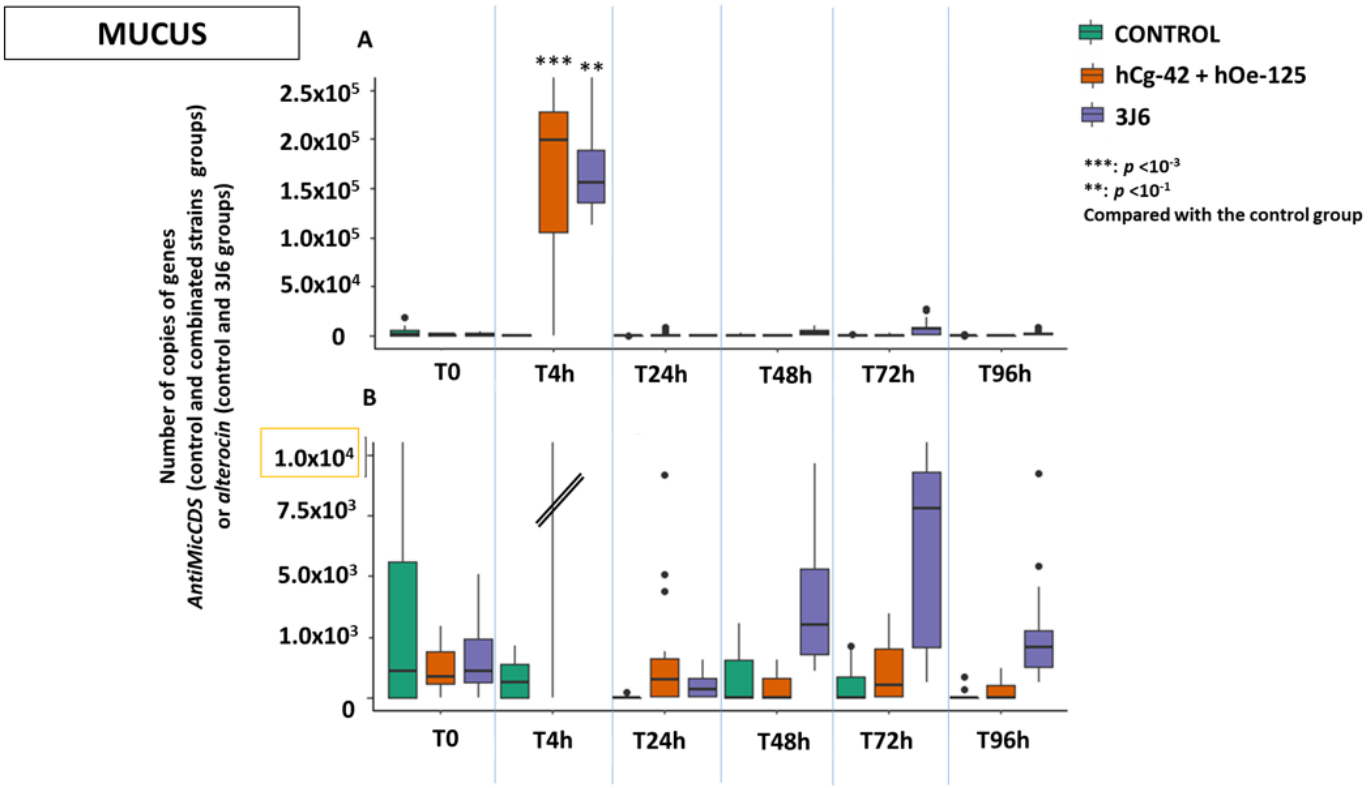

Figure 4

Copy numbers of the *AntiMicCDS* (representing strains h*Cg*-42 and h*Oe*-125) or *alterocin* (representing strain 3J6) genes in the mucus at T0 (before the immersion), after 4 h of immersion (T4H) and at 24 h, 48 h, 72 h and 96 h post-immersion for the control and probiotic treatments. (A): synthesis for all treatments tested. (B): identical data but with a modified scale more adapted to compare times other than T4H. **: $p \leq 10^{-2}$; ***; $p \leq 10^{-3}$

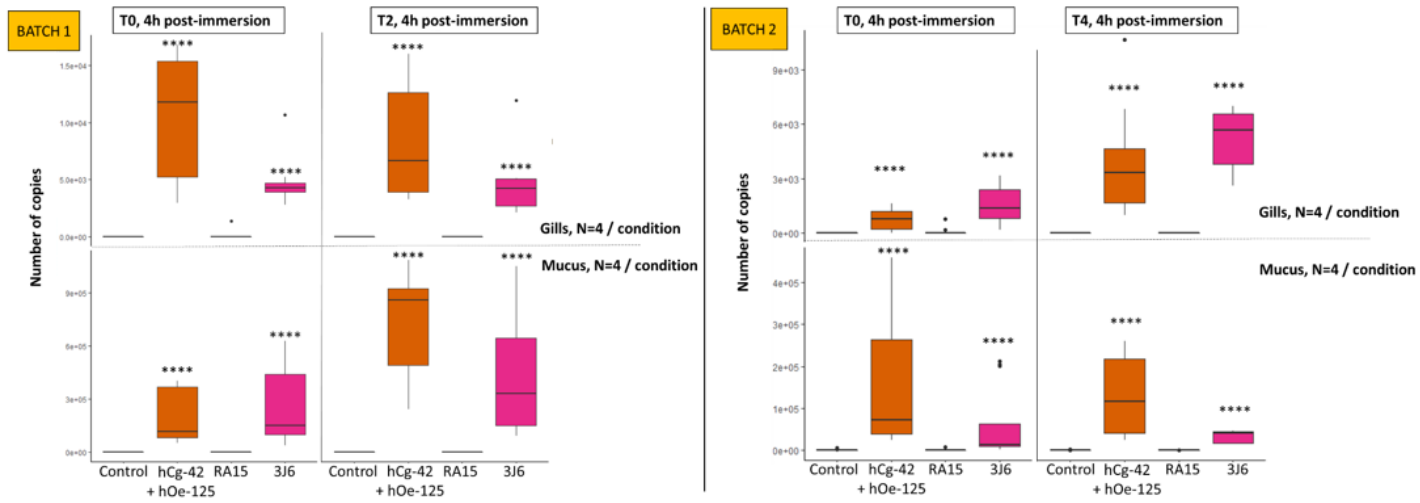

Figure 5

Copy numbers of *AntiMicCDS* (representing strains h*Cg*-42 and h*Oe*-125) or *alterocin* (representing strain 3J6) genes in gills and mucus after 4 h of immersion at T0 and T2 (Batch 1) and at T0 and T4 (Batch 2) for control and probiotic treatments. The *AntiMicCDS* gene was amplified for the control, RA15 and h*Cg*-42+h*Oe*-125 groups. The *alterocin* gene was amplified for the control, RA15 and 3J6 groups. ****: $p \leq 10^{-4}$

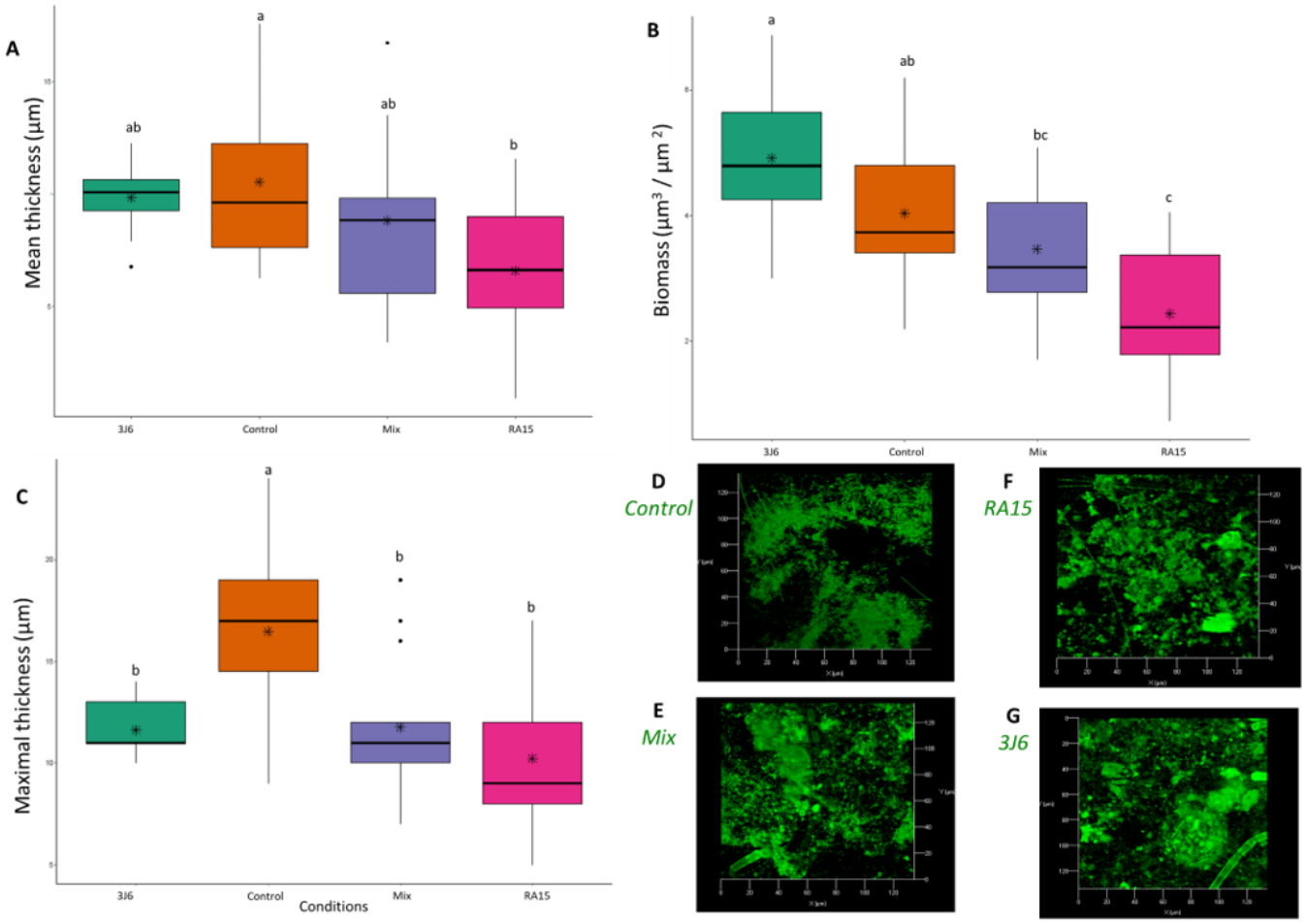

Figure 6

Impact of probiotics on physical biofilm parameters. Slides were collected during the impregnation of Batch 2. A total of four slides per treatment were analyzed (three for the RA15 treatment) and at least three images were taken for each slide. For each boxplot, different letters (a, b and c) indicate statistical differences between the treatments tested. $p < 0.05$

6A to 6C: Biofilm parameters. (A) Biofilm mean thickness in µm. (B) Biofilm biomass in $\mu m^3/\mu m^2$. (C). Maximal thickness in µm.

6D to 6G: Biofilms top views stained with SYTO-9 and observed by Confocal Laser Scanning Microscopy (obj 63x). (D) Non-impregnated control, (E) Combined strain, (F) RA15 strain, (G) 3J6 strain

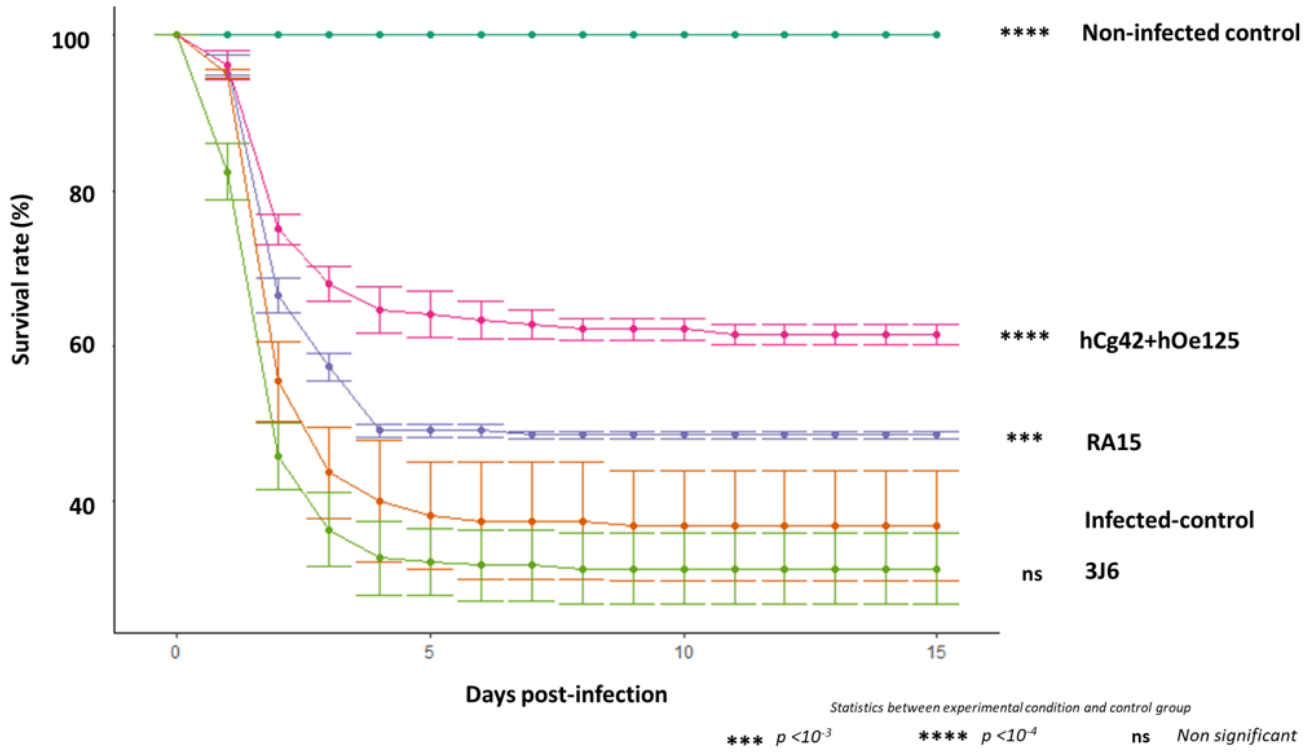

Figure 7

Survival rates of sea bass impregnated and non-impregnated with *Pseudoaltermonas* strains and infected by intraperitoneal injection with *Vibrio harveyi*. Survival rates are expressed as percentages and calculated during the 15 days post-infection (dpi). Statistics were performed to compare the infected control and the other groups (non-infected control, RA15, h*Cg*-42+h*Oe*-125 and 3J6). All treatments are composed of four biological replicates except for (i): the non-infected control (one biological replicate); (ii): the infected control (three biological replicates); (iii): the combined strains (three biological replicates). Each replicate was composed of 52 sea bass on average, from Batch 1. ns: non-significant; ***; $p \leq 10^{-3}$;**** : $p \leq 10^{-4}$

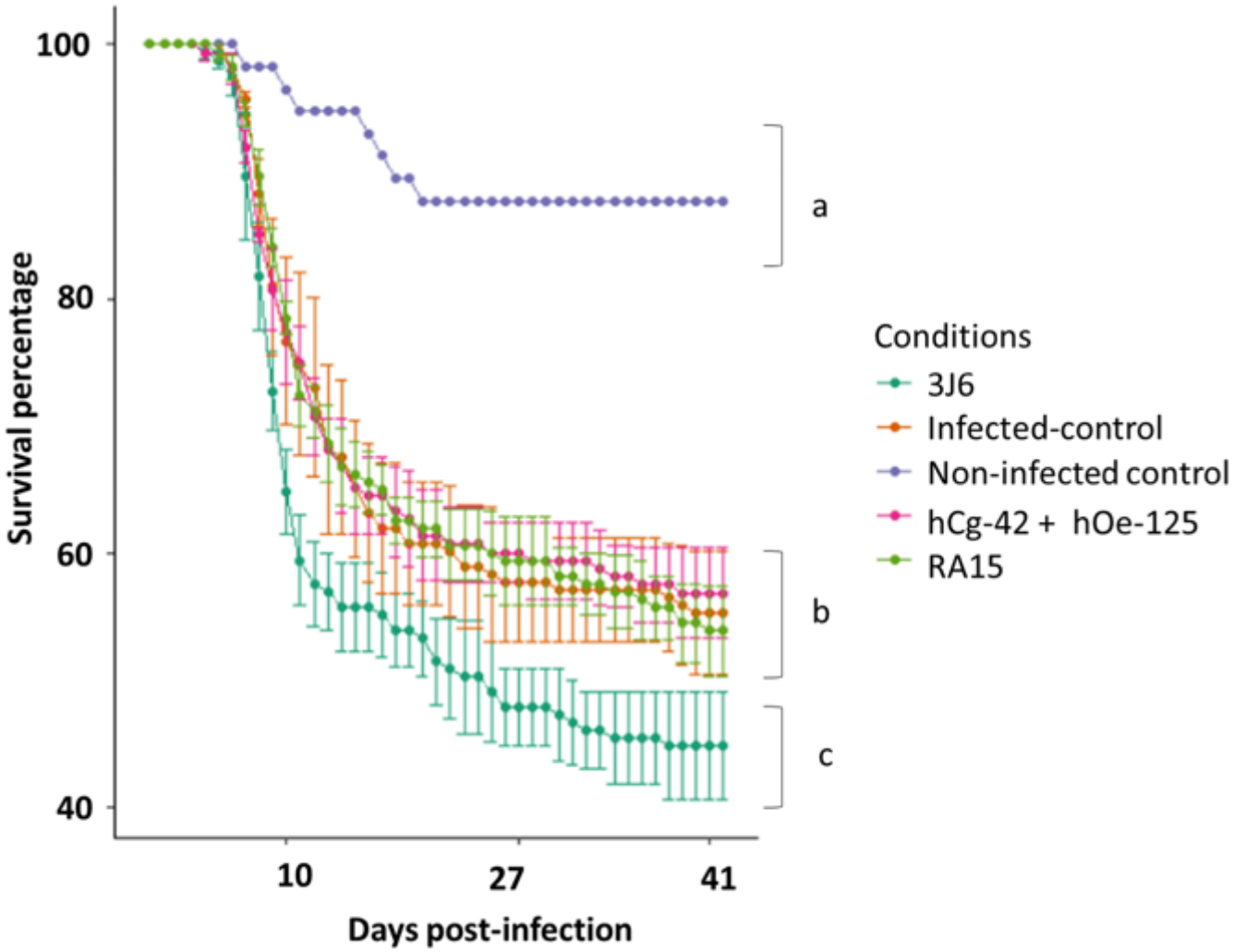

Figure 8

Survival rates of sea bass impregnated or non-impregnated with *Pseudoalteromonas* strains and infected with NNV. Survival rates are expressed as percentages and calculated during the 41 days post-infection (6 weeks). Statistics compare the infected control group and the other groups (non-infected control, RA15, h*Cg*-42+h*Oe*-125 and 3J6). Statistical groups are represented by letters a, b and c ($p \leq 10^{-4}$). All treatments are composed of three biological replicates. Each replicate was composed of 54 sea bass on average from Batch 2

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1Rahmanietal.2023.tif
- FigS2Rahmanietal.2023.tif
- FigS3Rahmanietal.2023.tif

- FigS4Rahmanietal.2023.tif
- FigS5Rahmanietal.2023.tif
- FigS6Rahmanietal.2023.tif