



Isolation and Characterization of Fish-Gut *Bacillus* spp. as Source of Natural Antimicrobial Compounds to Fight Aquaculture Bacterial Diseases

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Abstract

Aquaculture is responsible for more than 50% of global seafood consumption. Bacterial diseases are a major constraint to this sector and associated with misuse of antibiotics, pose serious threats to public health. Fish-symbionts, co-inhabitants of fish pathogens, might be a promising source of natural antimicrobial compounds (NACs) alternative to antibiotics, limiting bacterial diseases occurrence in aquafarms. In particular, sporeforming *Bacillus* spp. are known for their probiotic potential and production of NACs antagonistic of bacterial pathogens and are abundant in aquaculture fish guts. Harnessing the fish-gut microbial community potential, 172 sporeforming strains producing NACs were isolated from economically important aquaculture fish species, namely European seabass, gilthead seabream, and white seabream. We demonstrated that they possess anti-growth, anti-biofilm, or anti-quorum-sensing activities, to control bacterial infections and 52% of these isolates effectively antagonized important fish pathogens, including *Aeromonas hydrophila*, *A. salmonicida*, *A. bivalvium*, *A. veronii*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela*, *Tenacibaculum maritimum*, *Edwardsiella tarda*, and *Shigella sonnei*. By in vitro quantification of sporeformers' capacity to suppress growth and biofilm formation of fish pathogens, and by assessing their potential to interfere with pathogens communication, we identified three promising candidates to become probiotics or source of bioactive molecules to be used in aquaculture against bacterial aquaculture diseases.

Keywords Fish diseases · Sporeformers · Bioactivity · Biofilms · Quorum-quenching

Introduction

Aquaculture industry is the world's fastest growing animal protein producer. It supplies already 50% of the global seafood consumption, and it is indispensable to satisfy the

world's growing fish demand. However, frequent bacterial diseases occurring during fish farming can limit the worldwide growth of this sector (Plumb and Hanson 2011; Munn 2005; Lafferty et al. 2015). In fact, according to the United Nations, bacterial diseases are a major constraint

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to the economical profitability of the aquaculture industry (WHO 2014; Lafferty et al. 2015).

Vibriosis (*Vibrio* spp.), photobacteriosis (*Photobacterium damsela* sp.), tenacibaculosis (*Tenacibaculum maritimum*), edwardsiellosis (*Edwardsiella tarda*), and furunculosis (*Aeromonas salmonicida*) are among the most common bacterial diseases known to affect important marine aquaculture fish species, such as turbot (*Scophthalmus maximus*), European seabass (*Dicentrarchus labrax*), Senegalese sole (*Solea senegalensis*), and gilthead seabream (*Sparus aurata*) (Menanteau-Ledouble et al. 2016; Janda and Abbott 2010; Frans et al. 2011; Avendaño-Herrera et al. 2006; Park et al. 2012; Lafferty et al. 2015; Plumb and Hanson 2011; Rivas et al. 2013; Andreoni and Magnani 2014). The symptomatology of these diseases include hemorrhagic septicemia, ulcers, gill inflammation, exophthalmia, multifocal necrosis, and white granulomas in the hematopoietic tissues (Menanteau-Ledouble et al. 2016; Janda and Abbott 2010; Frans et al. 2011; Avendaño-Herrera et al. 2006; Park et al. 2012; Andreoni and Magnani 2014). The exact pathogenetic mechanisms are not fully understood, but virulence factors of the reported Gram-negative bacteria, both extracellular (e.g., cytotoxins, proteases) and cell-wall associated (e.g., adhesins, invasins), are thought to play a prominent role in the development of diseases (Dallaire-Dufresne et al. 2014; Defoirdt 2013; Rasmussen-Ivey et al. 2016). Additionally, most of these pathogens form biofilms (mono- or multi- species communities attached to a surface and embedded in a polysaccharide matrix) that appear determinant for colonization and persistence inside the host (Avendaño-Herrera et al. 2006; Frans et al. 2011; Janda and Abbott 2010; Park et al. 2012; Defoirdt 2013; Wang et al. 2015; Jones and Oliver 2009; Andreoni and Magnani 2014). When associated in biofilms, bacteria are generally more resistant to antibiotics and innate immune defenses and also express more virulence factors as result of gene activation by quorum-sensing (Fey 2010; Defoirdt 2013; Moons et al. 2009). Quorum-sensing (QS) is the molecular strategy used by bacteria to monitor and regulate the size and density of their own population (Miller and Bassler 2001; Abisado et al. 2018). QS systems are mediated by small diffusible signalling molecules (e.g., acyl homoserine lactone, autoinducer-2) that regulate virulence, biofilm formation, inter- and intra-specific interactions, or antibiotic resistance (Miller and Bassler 2001; Chu and McLean 2016; Abisado et al. 2018).

Importantly, there is increasing evidence that *Aeromonas* spp., *Vibrio* spp., *Photobacterium damsela* sp., and *Edwardsiella* spp. might be responsible for emerging zoonoses which are dangerous for public health (Gauthier 2015; Khajanchi et al. 2010; Cabello et al. 2016; Igbinsosa 2016). The routes of human infection are considered to be water (swimming or drinking) and water-associated food

like fish, shellfish, and raw-edible products contaminated via irrigation. Aquaculture farmers are frequently exposed to fish; therefore, they are at higher risk of infection through open wounds, bites, or pincer injuries (Weir et al. 2012). Furthermore, the susceptibility of the abovementioned bacteria to traditional antibiotic treatments is not well documented and their resistance to different classes of antibiotics has been reported (Khajanchi et al. 2010; Gauthier 2015; Cabello et al. 2016; Igbinsosa 2016). In this context, it is urgent to find alternative solutions to antibiotics that assure an advanced and integrated healthcare for humans, animals, and environment.

One of the most promising health-promoting strategies to improve fish resistance to diseases is the use of probiotics, defined as “live organisms which when administrated in adequate amounts confer a health benefit on the host” (Food and Agriculture Organization of the United Nations 2001). Probiotic bacteria may contribute to the gut microbial balance, enhance the host immune responses, compete with pathogens for adhesion sites and nutrients, or produce natural antimicrobial compounds (NACs). NAC can be metabolites, peptides, or proteins and might inhibit pathogen growth, biofilm formation, and QS (Hong et al. 2005; Hai 2015; Verschuere et al. 2000). Probiotic bacteria have also been implicated in bioremediation and water quality improvement and have been reported to reduce antibiotic residues in the environment, contributing to the sustainability of aquaculture (Verschuere et al. 2000).

Among bacteria currently used as probiotics, sporeforming species such as *Bacillus* spp. exhibit critical advantages for application in aquafeeds, as their spores are easily produced in large scale and can be dehydrated, thus facilitating feed incorporation and long-term storage without losing characteristics (Hong et al. 2005; Cutting 2011). Moreover, *Bacillus* spp. spores survive and transit through the gut since they are acid and bile tolerant (Tam et al. 2006). Further, *Bacillus* spp. are recognized to be NAC producers and are capable to antagonize important human and animal pathogens (Abriouel et al. 2011; Sahoo et al. 2016; Sumi et al. 2014). Indeed, we have recently isolated from the gut of European seabass sporeforming *Bacillus* spp. with promising probiotic characteristics, including a broad capacity to inhibit the growth of different fish pathogens (Serra et al. 2019). Thus, *Bacillus* spp. might be an ideal source of new NACs with anti-growth, anti-biofilm, or anti-QS activities, and thus a useful weapon against fish pathogens.

Here, we describe the isolation, identification, and characterization of sporeforming *Bacillus* spp. from the gut of fish with different feeding habits, and thus different gastrointestinal structures: the European seabass (*Dicentrarchus labrax*), white seabream (*Diplodus sargus*), and gilthead seabream (*Sparus aurata*). The bacterial isolates and their NACs were tested in vitro for their capacity

to suppress growth and biofilm formation of fish pathogens, their potential to interfere with pathogens QS, and their suitability to become probiotics for aquaculture.

Material and Methods

Experimental Conditions

The trial was performed at the experimental facilities of the Marine Zoological Station, Faculty of Sciences, Porto University. White seabream (*Diplodus sargus*) and European sea bass (*Dicentrarchus labrax*) juveniles were obtained from IPMA, Olhão, Portugal, and gilthead seabream (*Sparus aurata*) juveniles were obtained from Atlantik Fish, Algarve, Portugal. After 15 days of quarantine, fish were transferred to the experimental tanks and acclimatized to the rearing conditions for 15 days. The experiment was performed in a recirculating water system equipped with 9 cylindrical fiberglass tanks of 100-L capacity and thermo-regulated to 22.0 ± 1.0 °C. Tanks were supplied with continuous flow of filtered seawater ($2.5\text{--}3.5$ l min⁻¹), salinity average 34.0 ± 1.0 g l⁻¹, and dissolved oxygen was kept near saturation (7 mg l⁻¹). Seawater was filtered by passing through a biological filter followed by a sand filter and an UV light. Triplicate groups of 10 gilthead seabream (initial body weight (IBW) of 62 g), European seabass (IBW of 30 g), and white seabream (IBW of 40 g) were randomly distributed to each tank. The trial lasted 6 weeks, and during that period, fish were fed by hand twice daily, 6 days a week, until apparent visual satiation with a commercial diet (Skretting, Stavanger, Norway) containing 16% lipids and 47% protein. Fish handling and procedures were performed in compliance with the recommendations of the EEC Directive (2010/63/EU) for care and use of laboratory animals.

Sampling

At the end of the trial, nine fish of each species (3 fish per tank) were randomly captured 4 h after the morning meal (to guarantee that intestines were filled with digesta) and euthanized by a lethal dose (1 mL/L) of anesthetic (ethylene glycol monophenyl ether). Digesta contents were collected under aseptic conditions by squeezing the entire gut (without pyloric caeca), previously excised. To reduce inter-fish variation, each sample was composed of a pool of gut digesta contents from the 3 fish per tank.

Isolation, Selection, and Characterization of Sporeforming Bacteria

To select the aerobic bacterial sporeforming isolates, around 150 mg of each digesta were diluted in buffered peptone

water (0.9%) and homogenized by vigorous vortexing. Then, serial dilutions (10^{-0} , 10^{-1} , 10^{-2}) of homogenate were prepared in Bott and Wilson (B&W) salts (1.24% K₂HPO₄, 0.76% H₂PO₄, 0.1% trisodium citrate, 0.6% [NH₄]₂SO₄, pH6.7) and 100 µL aliquots were spread onto Luria Bertani (LB) agar plates (Becton, Dickinson and Company, USA) after 20-min heat treatment at 65 °C (Barbosa et al. 2005).

Following incubation at 37 °C for 48 h, colonies obtained from each digesta were counted and randomly selected by their different morphologies. All selected isolates were purified, numbered, and stored at -80 °C in 30% glycerol until used. Spore production was evaluated by phase-contrast microscopy observation of isolates grown for 48 h in solid Difco Sporulation Medium or DSM (Becton, Dickinson and Company, USA) (Barbosa et al. 2005), using a Nikon Eclipse Ci-L microscope, equipped with a CoolLED's pE 300 lite illumination system, and images were obtained with a DS-Ri 2 camera. All isolates were further investigated for catalase activity by suspending a fresh colony in 3% hydrogen peroxide (H₂O₂) solution; the production of air bubbles was considered a positive response (Barbosa et al. 2005).

Molecular Identification and Phylogenetic Analysis

Genomic DNA of sporeformers was extracted from overnight LB liquid cultures, based on the method of Pitcher et al. (1989) with few modifications. Briefly, each bacterial pellet was homogenized with 50 mg/mL of lysozyme (Sigma-Aldrich, Germany), in TE buffer (10 mM Tris HCl, 1 mM EDTA) followed the addition of by 2 mg/mL of RNase (Sigma-Aldrich, Germany). After incubation at 37 °C for 1 h, 10% SDS, and 0.2 mg/mL of proteinase K (Sigma-Aldrich, Germany) were added, and tubes incubated for 30 at 55 °C. Then, GES solution (5 M guanidine thiocyanate, 0.5 M EDTA, 10% N-lauroylsarcosine) and 7.5 M ammonium acetate precipitated the remaining proteins prior to nucleic acids extraction with of phenol:chloroform:isoamyl-alcohol (25:24:1). After the aqueous phase collection, the addition of chloroform:isoamyl-alcohol (24:1) allowed a re-extraction of the nucleic acids. The DNA was precipitated with 0.6 volumes of isopropanol washed with ice-cold 70% ethanol and dissolved in ultrapure water.

PCR amplification of the 16S rRNA gene was accomplished using universal primers 16S-27F (Weisburg et al. 1991) and 16S-1492R (Weisburg et al. 1991), at an annealing temperature of 55 °C. Each 50 µL reaction contained 1 × DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each dNTP (Thermo Scientific, Vilnius, Lithuania), 0.2 µM of each primer (STAB Vida, Lisboa, Portugal), 1.25 U of DreamTaq

DNA polymerase enzyme (Thermo Scientific, Vilnius, Lithuania), and 25 ng DNA template. The amplified products were sequenced with the primer 16S-27F (Weisburg et al. 1991) at STABVIDA (Caparica, Portugal).

Phylogenetic analysis was accomplished online, using the Sequence Match software package through the GenBank non-redundant nucleotide database with BLAST (<http://www.ncbi.nlm.nih.gov>) or by comparison with sequences in the Ribosomal Database Project 10 (<http://rdp.cme.msu.edu/>).

Bacterial Strains and Culture Conditions

The sporeforming bacterial strains isolated from fish gut were denominated “producer strains” and were routinely grown in LB medium at 37 °C, with constant agitation (120 rpm). The laboratory strain *Bacillus subtilis* 168 (Zeigler et al. 2008) (Table 1) was used as control for “producer strains”, and the Gram-positive *Staphylococcus aureus* LMG 2884, was used as control for “indicator strains” due to its inhibition by *B. subtilis* 168 which has been previously described (Serra et al. 2019). These controls were grown in the same conditions as the “producer strains”

and “indicator strains,” respectively. Target fish pathogens presented in Table 1 were used as “indicator strains” and were grown aerobically in BHI medium (except for *T. maritimum*, which was grown in Marine medium) at 25 °C (*A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Photobacterium damsela* subsp. *damsela*, *Photobacterium damsela* subsp. *piscicida*, and *T. maritimum*) or 37 °C (for *E. tarda*, *S. sonnei*, and *S. aureus*).

A strain of *Chromobacterium violaceum* (CECT 494) and the *cvil::mini-Tn5* mutant of *Chr. violaceum* CV026 (CECT 5999) (Table 1) were used as biosensors for quorum-quenching (QQ) detection. Biosensors were routinely cultivated aerobically in LB media at 30 °C for 48 h. *Pseudomonas aeruginosa* PAO-1 was used as positive control for QQ activity and was grown aerobically at 37 °C for 24 h.

Anti-growth Activity Screening

The antimicrobial activity of all producer strains was first evaluated by colony overlay assay, as described by Barbosa

Table 1 Bacterial strains used in the present study

Bacterial species	Strain	Origin/source
Fish pathogens		
<i>Aeromonas salmonicida</i>	LMG 3780	BCCM/LMG
<i>Aeromonas veronii</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas bivalvium</i>	Fish isolate	NUTRIMU collection
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	LMG 2844	BCCM/LMG
<i>Vibrio anguillarum</i>	DSM 21597	DSMZ
<i>Vibrio harveyi</i>	Fish isolate	NUTRIMU collection
<i>Vibrio parahaemolyticus</i>	LMG 2850	BCCM/LMG
<i>Vibrio vulnificus</i>	LMG 13545	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	LMG 7892	BCCM/LMG
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	<i>Lg_{h41/01}</i>	(Diaz-Rosales et al. 2003)
<i>Tenacibaculum maritimum</i>	LMG 11612	BCCM/LMG
<i>Edwardsiella tarda</i>	LMG 2793	BCCM/LMG
<i>Shigella sonnei</i>	LMG 10473	BCCM/LMG
Other		
<i>Staphylococcus aureus</i>	LMG 8224	F. Tavares
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	168 (BGSC1A1)	A.O. Henriques
<i>Chromobacterium violaceum</i> WT	CECT 494	CECT
<i>Chromobacterium violaceum</i> CV026	CECT 5999	CECT
<i>Pseudomonas aeruginosa</i> PAO-1	LMG 12228	F. Tavares

Bacterial strains were obtained from bacterial collections (BCCM/LMG, Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University, Ghent, Belgium; DSMZ, DSM Collection, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; CECT, Spanish Type Culture Collection, Valencia, Spain), from our laboratory stocks (NUTRIMU collection) or kindly supplied by F. Tavares (Centro de Investigação em Biodiversidade e Recursos Genéticos), M.A. Morinigo (Universidad Málaga), and A. O. Henriques (Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal)

et al. (2005). Briefly, producer strains were inoculated as 5 μL spots on LB plates, grown for 24 h and then killed with chloroform vapors. Plates were overlaid with soft BHI agar inoculated with the indicator strains ($\text{OD}_{600} \sim 0.1$) and incubated for 24 h at 25 °C or 37 °C (for *S. sonnei* and *E. tarda*). Growth inhibition zones around the colonies, after 24-h incubation at 25 °C or 37 °C as described above, were considered positive and the corresponding growth inhibition halos were measured (mm). All plates were photographed in Gel-Doc™ XR⁺ System, using Image Lab™ Software (Bio-Rad, EU).

The antimicrobial activity of cell-free supernatant containing NACs of the most promising producer strains (those exhibited a stronger antimicrobial activity against fish pathogens or “indicator strains”) was detected by two independent growth inhibition assays. First, a well-diffusion assay was performed as described by Serra et al. (2019). The indicator strains were inoculated ($\text{OD}_{600} \sim 0.1$) on BHI agar plates, assuring uniform coverage. Then, 9-mm-diameter wells were punched and filled with 100 μL cell-free supernatant of each producer strain, previously grown overnight at 37 °C and 150 rpm, centrifuged for 10 min at 11,800g, and sterilized by filtration with 0.22 μm cellulose acetate filter (VWR, Portugal). Growth inhibition zones around the wells after 24-h incubation at 25 °C or 37 °C were considered positive, and the corresponding growth inhibition halos were measured (mm). Plates were photographed as described above. For the second growth inhibition assay (microplate assay), 100 μL cell-free supernatant of each producer strain (obtained as described above) were added to 96-well flat-bottomed polystyrene plates and inoculated with 100 μL of indicator bacterial cultures ($\text{OD}_{600} \sim 0.1$). A positive control containing 100 μL of LB medium and 100 μL of the appropriate bacterial cultures ($\text{OD}_{600} \sim 0.1$) and a negative control containing only 200 μL of LB medium were also prepared. The 96-well microplate was aerobically incubated (120 rpm) at 25 °C, and the optical density at 600 nm (OD_{600}) was measured every hour for 24 h in a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.). The data is composed by three independent experiments.

Anti-biofilm Formation Screening

The ability of sporeformers to inhibit biofilm formation of each pathogenic strain was tested by a modification of Papa et al. (2015) method. In brief, 100 μL of indicator cultures ($\text{OD}_{600} \sim 0.1$) were added to 96-well flat-bottomed polystyrene plates. Each well was filled with 100 μL of cell-free supernatant of each sporeforming strain. Moreover, 100 μL of LB medium and 100 μL of the appropriate bacterial cultures ($\text{OD}_{600} \sim 0.1$) was used as positive control, and 200 μL of LB medium was used as negative control. After 24 h of aerobic incubation at 25 °C, bacterial cells were removed and the wells were washed 3 times with 250 μL of phosphate-buffered saline (PBS) and

allowed to dry in inverted position. The wells were stained with 250 μL of 0.1% crystal violet for 15 min at room temperature and rinsed twice with 250 μL of double-distilled water. After wells' drying (in inverted position), the dye bound to adherent cells was solubilized in 250 μL of 20% glacial acetic acid and 80% ethanol for 30 min at room temperature. The final quantification of biomass was accessed by measuring the optical density at 590 nm in a Microplate Spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific Inc.). The data is composed by three independent experiments.

Anti-quorum Sensing (Anti-QS) Screening (Quorum-Quenching-Potential)

Sporeformers QQ potential was evaluated as described by McLean et al. (2004) with few modifications. Briefly, producer strains were inoculated as 5 μL spots on LB plates and grown at 37 °C. After overnight incubation, bacterial spots were killed with chloroform vapors for 30 min, followed by plate covers replacement and plates aeration for 20 min. Then, producer strains were overlaid with LB soft agar (0.8% agar) previously inoculated with 100 μL of biosensor strains ($\text{OD}_{600} \sim 0.1$), followed by plates incubation for 48 h at 30 °C. In the case of *Chr. violaceum* CV026, the LB soft agar was supplemented with 25 $\mu\text{g}/\text{mL}$ of kanamycin (Nzytech, Lisboa, Portugal) and N-(β -ketocaproyl)-L-homoserine lactone (3-Oxo-C6-HSL) (Sigma-Aldrich, Germany) to 5 μM of final concentration (McCleary et al. 1997), prior to bacterial culture inoculation. A positive QQ result was represented by inhibition of purple pigmentation around the producer strains. The cell-free supernatant screening was performed by overlaying LB plates with LB soft agar (0.8% agar) previously inoculated with 100 μL of biosensor strains ($\text{OD}_{600} \sim 0.1$) in the same experimental conditions described above. Once the plates solidified, 9-mm-diameter wells were punched and filled with 100 μL cell-free supernatant of each producer strain (obtained as described above). Zones of purple pigmentation inhibition around the wells after incubation for 48 h at 30 °C were considered a positive result. *P. aeruginosa* PAO-1 and laboratory strain *B. subtilis* 168 were used as positive control for QQ activity and for bacterial growth, respectively. All digital photos were taken with a Sony IMX240 camera and zones (in mm) of pigment inhibition recorded.

Sporulation Capacity

Sporulation of each “producer strain” was induced by nutrient exhaustion in DSM (Difco Sporulation Medium) for 24 h at 37 °C in an orbital shaker at 150 rpm. Grown cultures were serially diluted in B&W isotonic buffer (Bott and Wilson salts: 1.24% K_2HPO_4 , 0.76% H_2PO_4 , 0.1% trisodium citrate, 0.6% $[\text{NH}_4]_2\text{SO}_4$, pH6.7), and plated in LB agar before and after 20 min heat treatment at 80 °C, to

determinate heat-resistant spores. After incubation at 37 °C for 24 h, visible colonies were counted and the sporulation efficiency determined by quantifying colony forming units (CFU/mL) titer before and after the heat treatment (Barbosa et al. 2005; Nicholson and Setlow 1990). The data is composed of three independent experiments.

Antibiotic Susceptibility Test

Antibiotic susceptibility of “producer strains” was determined by the disk diffusion method of Kirby-Bauer (Bauer et al. 1966), using antimicrobial susceptibility discs (Oxoid Limited, Thermo Fisher Scientific Inc.) containing teicoplanin 30 µg/disc (TEC₃₀), vancomycin 30 µg/disc (VA₃₀), chloramphenicol 30 µg/disc (C₃₀), tetracycline 30 µg/disc (TE₃₀), erythromycin 15 µg/disc (E₁₅), gentamycin 10 µg/disc (CN₁₀), kanamycin 30 µg/disc (K₃₀), and streptomycin 10 µg/disc (S₁₀). The antibiotics were tested followed the recommendations of the European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed (FEEDAP 2012). Bacterial inoculums were prepared in sterile saline solution, at an optical density adjusted to 0.5 McFarland standard units (OD₆₀₀~0.1) and were spread with cotton swab onto 20 mL MH (Muller-Hinton, Oxoid) agar plates, assuring full coverage, and the antibiotics were distributed on the plates with a disk dispenser (Oxoid Limited, Thermo Fisher Scientific Inc.). After 24 h of incubation at 37 °C, bacteria were classified as sensitive (S), intermediate (I), and resistant (R), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013).

Statistical Analysis

The statistical analysis was done using the SPSS 23.0 software package for Windows. Before any test, data were checked for normal distribution and subject to Levene’s test to ensure homogeneity of variances that complies the requirements of ANOVA.

Repeated measures ANOVA and one-way ANOVA were performed to evaluate differences in the ability of sporeforming isolates to inhibit pathogens growth and biofilm formation, respectively. When *p* values were significant (*p* < 0.05), means were compared with Dunnett’s test.

Results

Culturable Aerobic Sporeformers Are Abundant in the Gut of Marine Fish

The heat-treated gut content of each fish species allowed selection, isolation, and purification of 176 bacterial isolates (61 from gilthead seabream, 51 from white seabream, and

64 from European seabass). Strain’s selection was based on macroscopic differences in colony morphologies and also on the collection of samples from different animal groups (tanks). Spore production analysis, induced by nutrient exhaustion in DSM, revealed that 172 strains (98%) produced endospores of different sizes and shapes (Table 2 and Fig. 1), and were catalase positive, indicating that these were probably *Bacillus* spp. and not aero-tolerant strains of *Clostridium* spp. (catalase negative) (Table 2).

Bacillus Sporeformers from Fish-Gut Are Promising Antagonists of Fish Pathogens

Among the 172 endospore-forming fish isolates (FIs) evaluated for their antimicrobial activity against a wide range of fish pathogens, 52% (90 isolates) were able to inhibit at least one of the tested pathogenic strains, and 23% (41 isolates) inhibited 2 or more pathogens. The later 41 isolates were identified by partial sequencing of the 16S rRNA gene (~700 kb), revealing a clear abundance of *B. subtilis* (54%) among the sporeformers isolated from the gut of gilthead seabream, white seabream, and European seabass (Table 2). *B. licheniformis* and *B. methylotrophicus* represented 9% and *B. amyloliquefaciens* represented 7% of the identified strains, whereas other bacterial species were present in smaller quantities. The identification of bacterial species was inconclusive for 10% of the strains (Table 2).

The colony-overlay assay allowed a progressive selection of the 8 most promising fish isolates according to the size of the inhibitory halos as well as the number of fish pathogens that they inhibited. The 8 FIs with the most promising antimicrobial activities, namely FI314, FI330, FI347, FI359, FI368, FI376, FI442, and FI480, were then tested simultaneously (via colony-overlay assay), in the exact same conditions (Fig. 2). Strains FI314, FI330, and FI442 showed antagonistic capacity against the growth of all pathogenic strains, except for *S. sonnei*. FI347 was active against *A. hydrophila* and *A. veronii*. FI359 inhibited the growth of *A. hydrophila*, *A. veronii*, *V. harveyi*, *S. aureus* and, in a smaller extent, of *P. damsela* subsp. *piscicida*. FI368, FI376, and FI480 inhibited the growth of *S. aureus*, *V. harveyi*, and *A. veronii* and, less markedly, that of *P. damsela* subsp. *piscicida*.

Antimicrobial Activity of *Bacillus* Sporeformers Is Mediated by Extracellular NACs

The antimicrobial activity of cell-free supernatant of the most promising *Bacillus* sporeformers (FI314, FI330, FI347, FI359, FI368, FI376, FI442, and FI480) is illustrated in Fig. 3. The extracellular compounds (hereinafter referred as extracellular NACs) of FI314 and FI330 produced clear inhibitory halos

Table 2 Identification and characterization of the 41 sporeformers isolated from the intestinal contents of white seabream (WSB), gilthead seabream (GSB), and European seabass (ESB) with antimicrobial activity against 2 or more fish pathogens

Isolate ^a	Source	Tank	16S rRNA gene analysis ^b		GenBank accession	Sporulation test ^c	Catalase test ^d
			Closest known species	% ID			
FI300	GSB	1	<i>Bacillus sp.</i>	98	MT081444	+	+
FI302	GSB	1	<i>B. subtilis</i>	98	MT081445	+	+
FI304	GSB	1	<i>B. subtilis</i>	99	MT081446	+	+
FI307	GSB	2	<i>B. licheniformis</i>	98	MT081447	+	+
FI314	GSB	2	<i>B. subtilis</i>	97	MT081448	+	++
FI321	GSB	2	<i>Bacillus sp.</i>	99	MT081449	+	++
FI324	GSB	2	<i>B. cereus</i>	99	MT081450	+	++
FI326	GSB	2	<i>B. subtilis</i> ; <i>B. cereus</i>	99	MT081451	+	++
FI330	GSB	3	<i>B. subtilis</i>	98	MT081452	+	++
FI333	GSB	3	<i>B. methylotrophicus</i>	97	MT081453	+	+
FI335	GSB	3	<i>B. methylotrophicus</i>	98	MT081454	+	++
FI338	GSB	3	<i>B. subtilis</i>	98	MT081455	+	++
FI342	GSB	3	<i>B. licheniformis</i>	98	MT081456	+	++
FI347	GSB	3	<i>Bacillus sp.</i>	98	MT081457	+	+
FI348	GSB	3	<i>B. licheniformis</i>	99	MT081458	+	+
FI353	WSB	1	<i>B. subtilis</i>	96	MT081460	+	+
FI354	WSB	1	<i>B. subtilis</i>	98	MT081461	+	+
FI355	WSB	1	<i>B. subtilis</i>	98	MT081462	+	+
FI359	WSB	1	<i>B. subtilis</i>	98	MT081463	+	++
FI361	WSB	1	<i>B. licheniformis</i>	99	MT081464	+	++
FI367	WSB	2	<i>B. amyloliquefaciens</i> ; <i>B. methylotrophicus</i>	99	MT081465	±	+
FI368	WSB	2	<i>B. subtilis</i>	98	MT081466	+	+
FI373	WSB	2	<i>B. subtilis</i>	97	MT081467	+	+
FI375	WSB	3	<i>B. pumilus</i>	97	MT081468	+	+
FI376	WSB	3	<i>B. subtilis</i>	97	MT081469	+	++
FI377	WSB	3	<i>B. subtilis</i>	98	MT081470	+	++
FI378	WSB	3	<i>B. subtilis</i>	99	MT081471	±	+
FI387	WSB	3	<i>B. subtilis</i>	98	MT081472	+	++
FI390	WSB	3	<i>Bacillus sp.</i>	99	MT081473	+	+
FI401	ESB	1	<i>B. subtilis</i>	97	MT081474	+	+
FI414	ESB	1	<i>B. methylotrophicus</i>	99	MT081475	+	+
FI423	ESB	2	<i>B. amyloliquefaciens</i>	99	MT081476	+	++
FI424	ESB	2	<i>Bacillus sp.</i>	99	MT081477	+	+
FI429	ESB	2	<i>B. amyloliquefaciens</i>	100	MT081478	+	+
FI436	ESB	3	<i>B. subtilis</i>	98	MT081479	+	++
FI442	ESB	3	<i>B. subtilis</i>	98	MT081480	+	++
FI455	ESB	3	<i>B. subtilis</i>	97	MT081481	+	+
FI456	ESB	3	<i>B. subtilis</i>	99	MT081482	+	+
FI464	ESB	3	<i>B. safensis</i>	99	MT081483	+	+
FI469	ESB	3	<i>B. subtilis</i>	98	MT081484	+	+
FI480	ESB	3	<i>B. subtilis</i>	99	MT081485	+	+

^aIn bold are the 8 isolates with the most promising antimicrobial activities

^bClosest known species using BLAST based on partial sequences of 16S rRNA gene (~700nt)

^cSpores detected by phase-contrast microscopy of 24 h cultures in DSM agar; +extensive formation of spores; ± limited formation of spores

^dCatalase activity tested by resuspending a colony in a 3% solution of hydrogen peroxide (Sigma); ++ fast positive reaction; + slow positive reaction

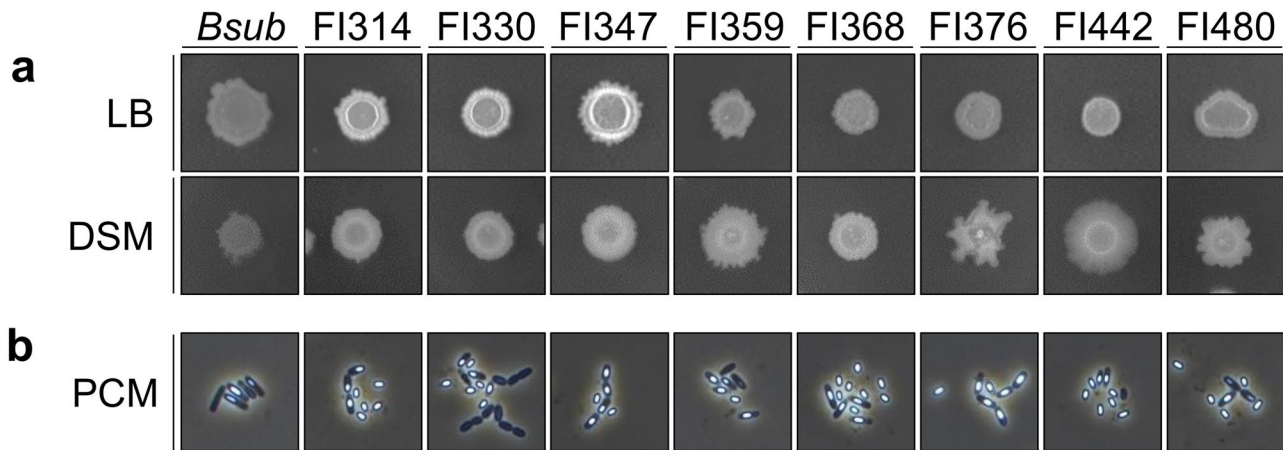


Fig. 1 **a** Morphological diversity of representative sporeforming isolates (FI numbers on top) obtained from fish intestinal contents. Photographs of colonies grown for 24 h at 37 °C in LB (Luria–Bertani) and DSM (Difco sporulation medium) agar medium were taken in a Gel-Doc™ XR+System, using the Image Lab™ Software (Bio-Rad, EU) and are at the same scale. **b** Sporulation capacity of the fish isolates, illustrating refractile sporulating cells and free spores.

against *A. salmonicida*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *P. damsela* subsp. *damsela*, *T. maritimum*, and *S. aureus*, and small inhibitory halos (2 mm, not clearly visible in Fig. 3) against *A. bivalvium*. FI442 extracellular NACs inhibited *V. vulnificus* and *P. damsela* subsp. *damsela*. The remaining strains (FI347, FI359, FI368, FI376, and FI480) did not inhibit the growth of the tested fish pathogens.

Based on the results of these experiments, six producer strains were selected having the best antimicrobial potential: FI314, FI330, FI359, FI376, FI442, FI480. The antimicrobial pattern of their extracellular NACs against the same fish pathogens was quantified via a growth-inhibition assay in liquid medium (microplate assay). FI314, FI330, and FI442 extracellular NACs were the most efficient in inhibiting bacterial growth (Fig. 4). In particular, the extracellular NACs of strains FI314 and FI330 reduced the growth of all the pathogens ($p < 0.001$). Furthermore, these two strains completely inhibited the growth of *A. salmonicida*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *P. damsela* subsp. *damsela*, *T. maritimum*, and *S. aureus* for the entire duration of the assay (24 h). The extracellular NACs of FI442 extended the lag phase duration of *V. anguillarum* and *P. damsela* subsp. *piscicida* growth curves, and completely inhibited the growth of *V. vulnificus* and *P. damsela* subsp. *damsela* for 24 h. In general, the NACs of this isolate reduced the growth of *A. salmonicida*, *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *T. maritimum*,

Spore production was evaluated by phase-contrast microscopy (PCM) using a Nikon Eclipse Ci-L microscope, equipped with a CoolLED's pE 300 lite illumination system, of isolates cultured for 48 h in solid DSM. Images were obtained with a DS-Ri 2 camera, all in the same conditions and at the same scale. The laboratory strain *B. subtilis* 168 (*Bsub*, on top) was used as a control

S. aureus, and *P. damsela* subsp. *damsela* ($p < 0.01$). The extracellular NACs of FI376 reduced the growth of *T. maritimum* and *S. aureus* ($p < 0.01$), whereas those of FI480 reduced the growth of *P. damsela* subsp. *damsela* and *T. maritimum* ($p < 0.01$). Interestingly, the growth of *A. salmonicida* and *V. harveyi* was higher in the presence of FI359 and FI376 ($p < 0.05$) cell-free supernatants when compared to the control. FI480 cell-free supernatant also increased *A. salmonicida* growth ($p < 0.05$).

Overall, only the fish isolates FI314, FI330, and FI442 were capable of suppressing fish pathogen's growth for 24 h in microplate (Fig. 4) produce clear inhibitory halos against the bacteria in the well agar diffusion assay (Fig. 3).

Extracellular NACs of *Bacillus* Sporeformers Inhibit Fish-Pathogen Biofilms

Cell-free supernatants of the six isolates tested above (FI314, FI330, FI359, FI376, FI442, FI480) were also evaluated for their ability to interfere with biofilm formation of each fish pathogen (Fig. 5). The extracellular NACs of FI314 and FI330 decreased the biofilm formation of *A. salmonicida*, *V. anguillarum*, *V. parahaemolyticus*, *P. damsela* subsp. *damsela*, *T. maritimum*, and *S. sonnei* ($p < 0.01$). FI330 also reduced *A. hydrophila* biofilm ($p < 0.05$). The extracellular NACs of FI359 and FI376 were active on biofilms of *A. hydrophila*, *A. salmonicida* and *S. sonnei*, whereas those of FI442 and FI480 reduced the biofilm synthesis of *A. salmonicida* and *S. sonnei* ($p < 0.05$). The control *B. subtilis* 168 was also able to decrease the biofilm synthesis of *T. maritimum* ($p < 0.05$).

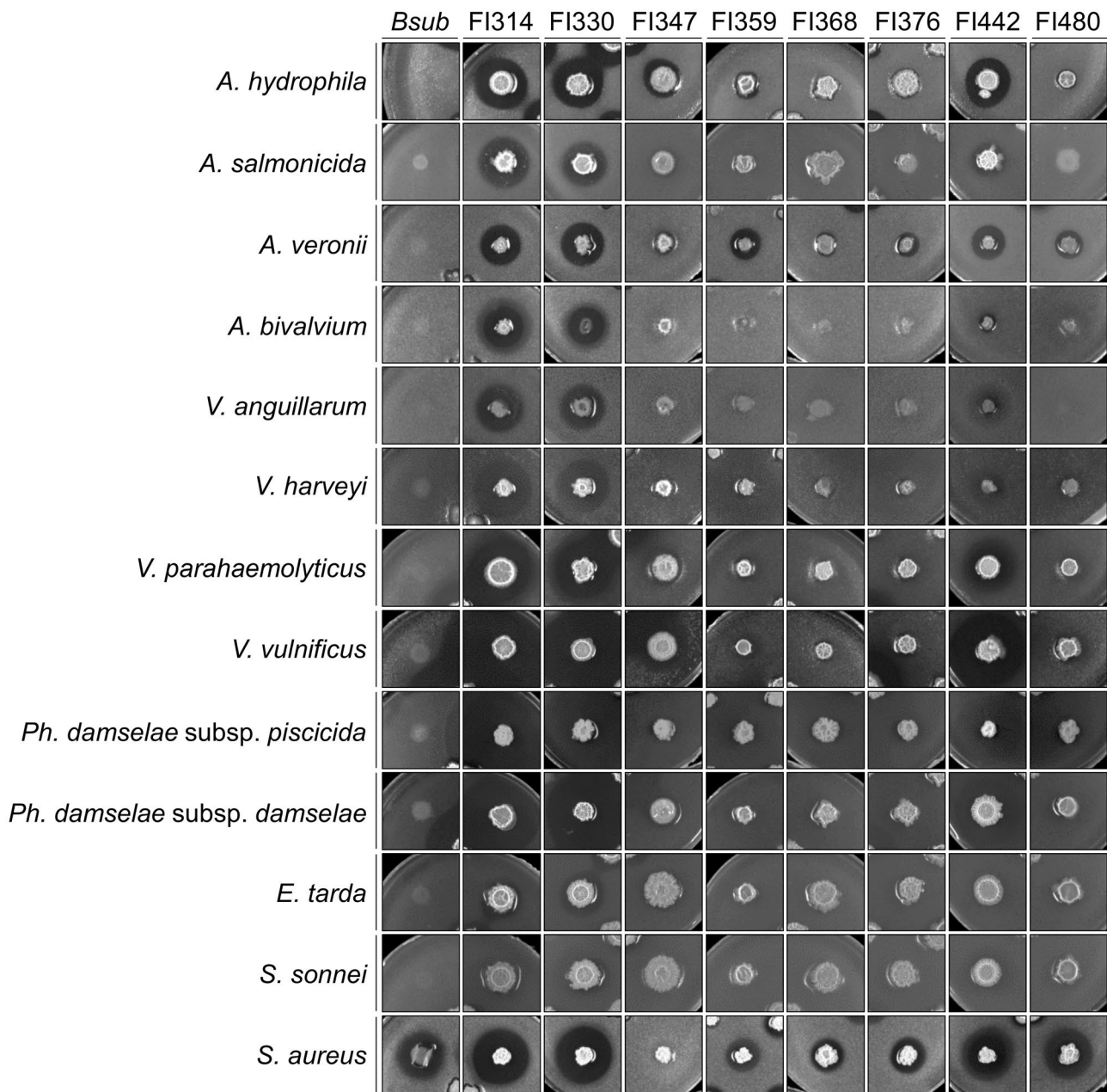


Fig. 2 Growth inhibition zones for the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *P. damsela* subsp. *damselae*, *E. tarda*, *S. sonnei*, and *S. aureus* around

colonies of sporeforming fish isolates (FI numbers on top). The laboratory strain *B. subtilis* 168 was used as a control. All photos were taken in a Gel-Doc™ XR + System, using the Image Lab™ Software (Bio-Rad, EU) and are at the same scale

Extracellular NACs of *Bacillus* Sporeformers Have QQ Activity

Chr. violaceum WT CECT 494 produces and senses several AHLs (e.g., N-(3-hydroxydecanoyl)-L-homoserine lactone and N-octanoyl-L-Homoserine lactone), whereas *Chr. violaceum* CV026 is unable to synthesize AHL, but responds to exogenous AHLs supplementation.

QQ was confirmed by the loss of purple violacein pigmentation around the sporeforming strains FI314, FI330, and FI442, suggesting the inactivation of AHLs produced by *Chr. violaceum* WT CECT 494 and of the 3-oxo-C6-HSL signal molecule externally added to *Chr. violaceum* CV026 biosensor (Fig. 6a). Additionally, the observed QQ activity of FI314 and FI330 was due to extracellular NACs, as their cell-free supernatants displayed activity against 3-oxo-C6-HSL signal molecule and interfered with

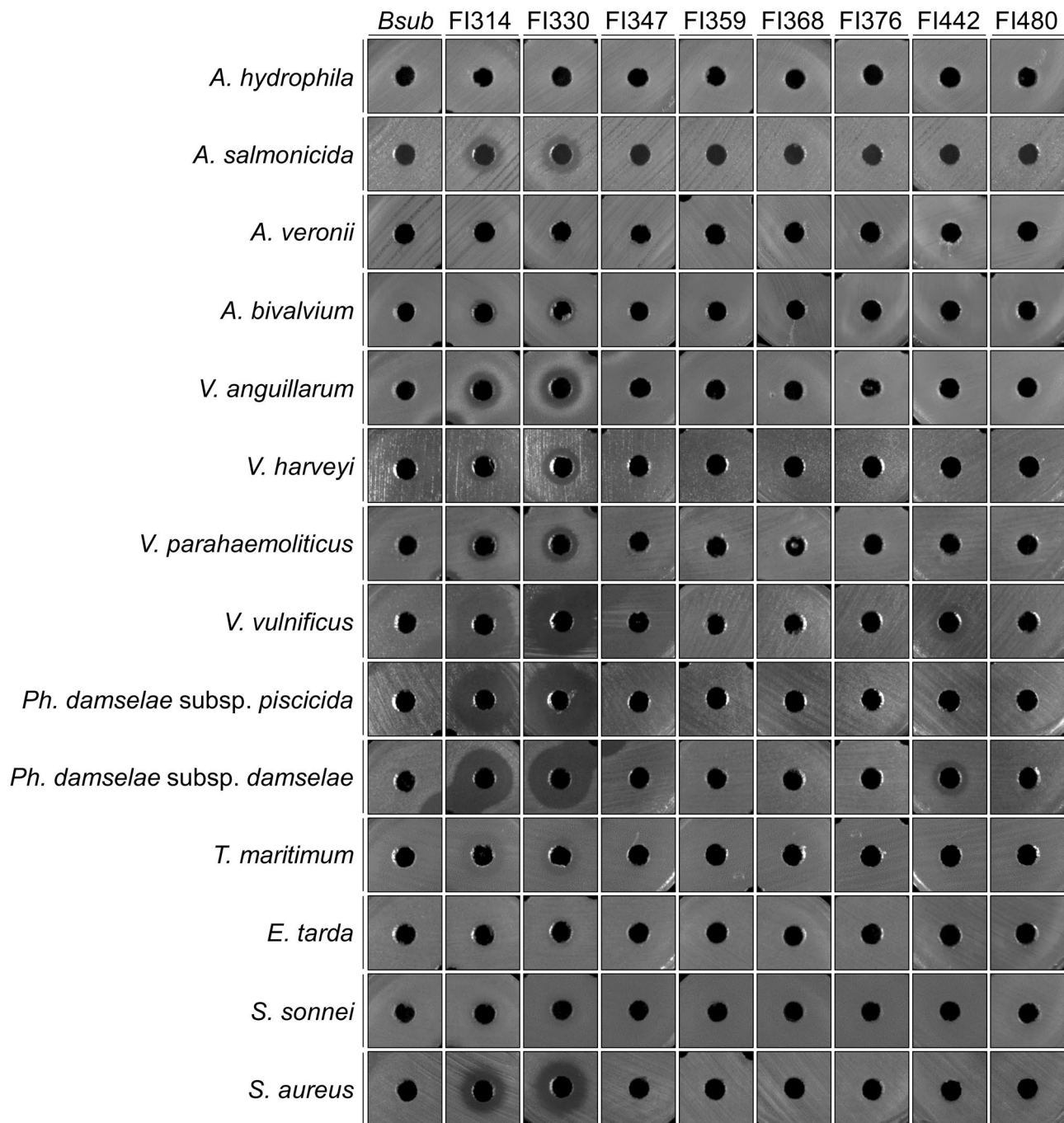


Fig. 3 Growth inhibition zones for the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela* subsp. *piscicida*, *P. damsela* subsp. *damsela*, *T. maritimum*, *E. tarda*, *S. sonnei*, and *S. aureus* around the wells with cell-free supernatant of sporeform-

ing fish isolates (FI numbers on top). All photos were taken in a Gel-Doc™ XR+ System, using the Image Lab™ Software (Bio-Rad, EU) and are at the same scale. The laboratory strain *B. subtilis* 168 (*Bsub* on top) was used as a control

the AHLs produced by *Chr. violaceum* WT CECT 494 to a lower extent (Fig. 6b). On the contrary, FI442 cell-free supernatant did not show any QQ activity. The control *P. aeruginosa* PAO-1 effectively degraded the AHLs tested in all the experiments performed during the present study.

Most Promising *Bacillus* Fish-Gut Sporeformers Have Probiotic Potential

Sporulation efficiency of the most promising strains (FI314, FI330, FI442) was determined due to their potential

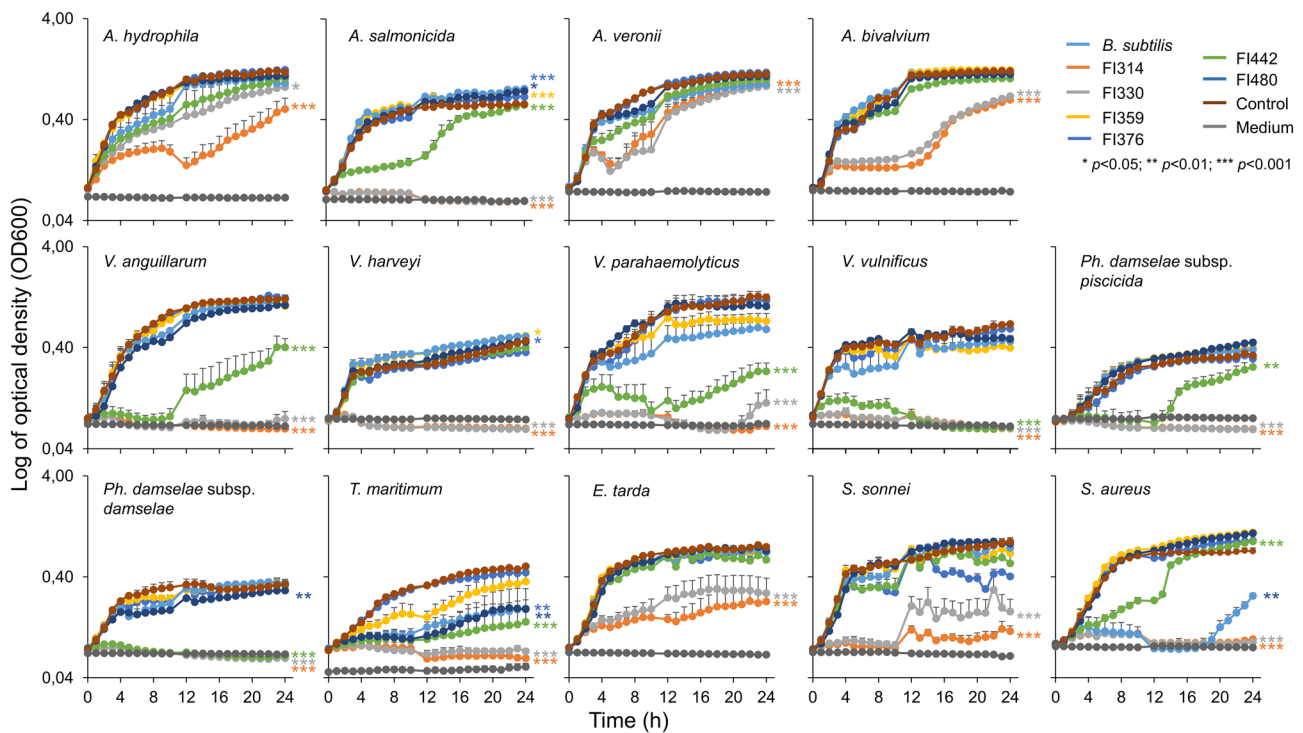


Fig. 4 Growth inhibition assays of the indicator strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela subsp. piscicida*, *P. damsela subsp. damsela*, *T. maritimum*, *E. tarda*, *S. sonnei*, and *S. aureus* when cultured alone in brain heart infusion (BHI) or marine (MA) medium (in the case of *T. maritimum*), or supplemented with cell-free supernatant of the sporeforming isolates (FI numbers). Pathogenic strains growing without cell-

free supernatant's supplementation were used as positive control (control), and BHI or MA medium alone used as negative control (medium). The laboratory strain *B. subtilis* 168 was used as a control for bacterial growth. The data are obtained from three independent experiments. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively)

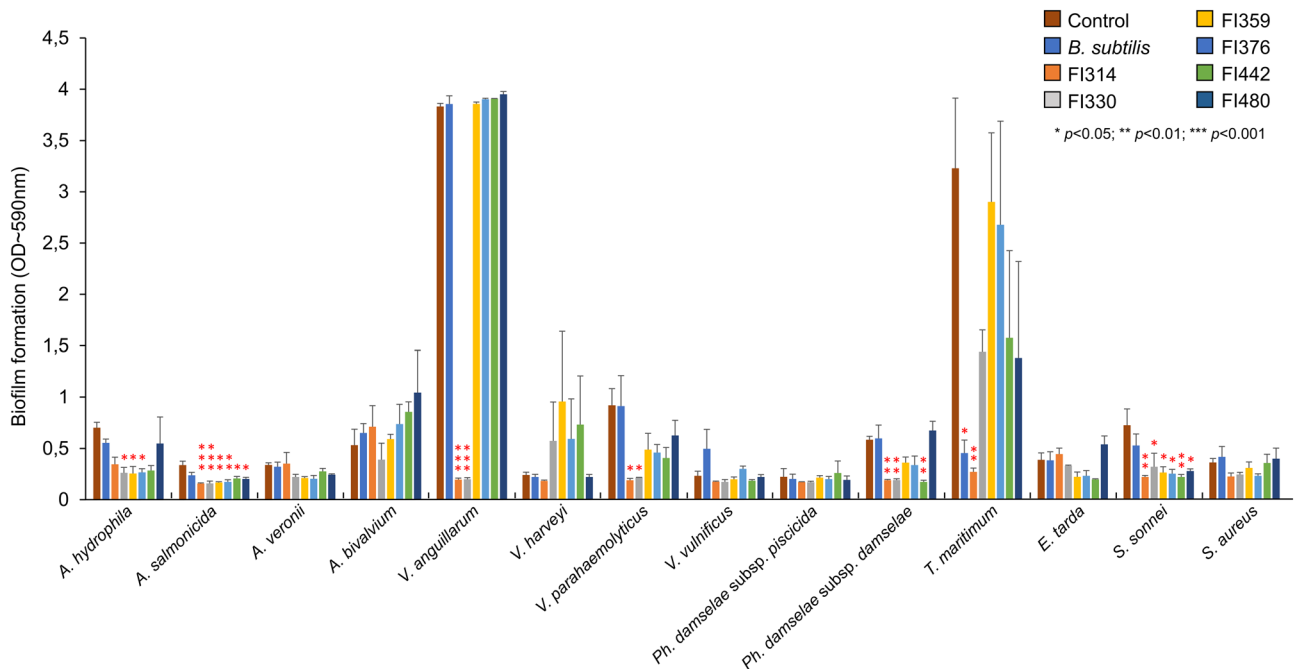


Fig. 5 Biofilm formation of the fish pathogens *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. damsela subsp. piscicida*, *P. damsela subsp. damsela*, *T. maritimum*, *E. tarda*, *S. sonnei*, and *S. aureus* when cultured alone in brain heart infusion (BHI) or marine (MA) medium (in the case of *T. maritimum*), or supplemented with

cell-free supernatant of the sporeforming isolates (FI numbers). The laboratory strain *B. subtilis* 168 was used as a control for bacterial growth. The data are obtained from three independent experiments. Significant differences ($p < 0.05$; $p < 0.01$; $p < 0.001$) in relation to control are represented by asterisks (*, **, ***, respectively)

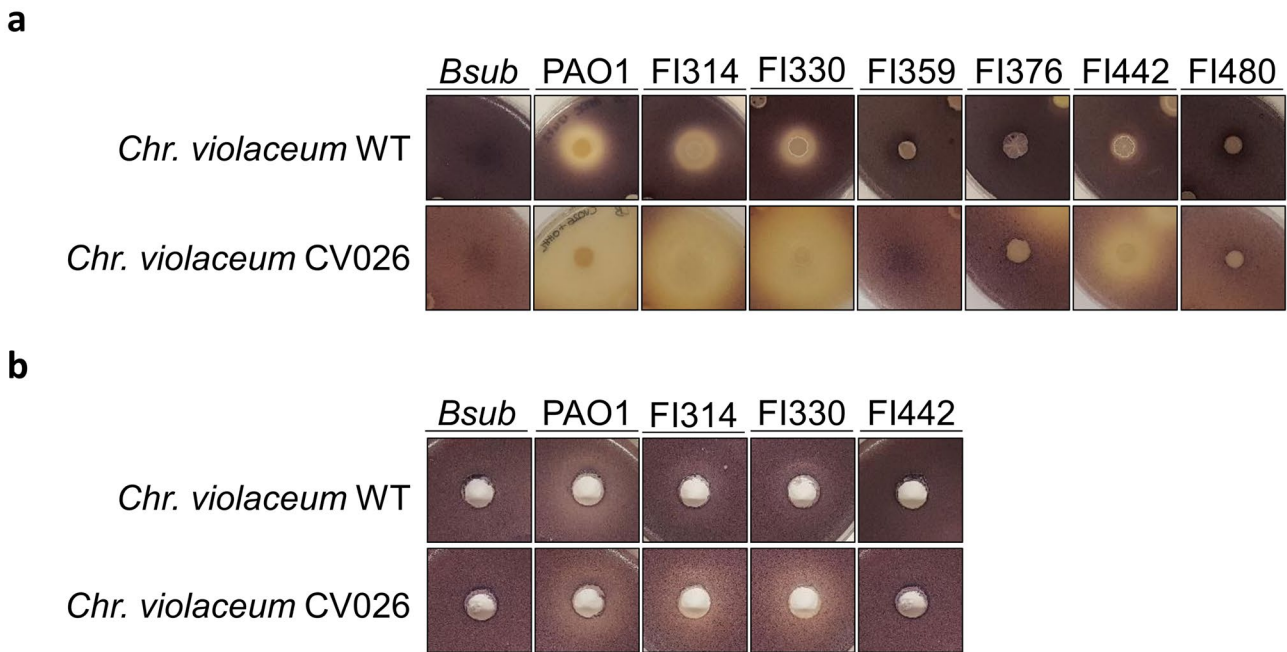


Fig. 6 Degradation of the violacein pigment produced by *Chr. violaceum* biosensors around colonies of sporeforming fish isolates **a** or around the wells with cell-free supernatant of sporeforming fish isolates **b**. The laboratory strain *B. subtilis* 168 (*Bsub*) was used as a

control for bacterial growth and *P. aeruginosa* PAO-1 as control for quorum-quenching (QQ) activity. All photos were taken with a Sony IMX240 camera and are at the same scale

industrial application as probiotics. By comparison with the laboratory strain *B. subtilis* 168 (Zeigler et al. 2008), all the selected strains showed good spore titres (around 10^8 CFU/mL), and sporulating efficiency $\geq 79\%$ (Table 3). All

“producer trains” were also sensitive to all the antibiotics required by EFSA (FEEDAP 2012), with the exception of FI314 isolate that presented an intermediate susceptibility to streptomycin (S_{10}) (Table 3).

Table 3 Sporulating efficiency and antibiotic susceptibility profile to antibiotics recommended by the European Food Safety Authority (EFSA) of the three most promising strains

	<i>B. subtilis</i>	FI314	FI330	FI442
Sporulation efficiency ^a				
Viable cells (CFU mL ⁻¹)	2.6×10^8	2.1×10^8	4.2×10^8	5.0×10^8
Heat-resistant spores (CFU mL ⁻¹)	1.8×10^8	2.4×10^8	3.8×10^8	3.9×10^8
Sporulation efficiency (%)	69	100	89	79
Antibiotic susceptibility profile ^b				
TEC ₃₀	S	S	S	S
VA ₃₀	S	S	S	S
C ₃₀	S	S	S	S
TE ₃₀	S	S	S	S
E ₁₅	S	S	S	S
CN ₁₀	S	S	S	S
K ₃₀	S	S	S	S
S ₁₀	S	I	S	S

^aSporulation was induced by nutrient exhaustion in DSM (Difco sporulation medium). At 24 h after inoculation, viable and heat-treated cells were measured by plating serial dilutions before and after 20-min heat treatment at 80 °C, respectively. Sporulation efficiency was calculated as the quotient between the heat-resistant cells and the total viable cells

^bStrain’s susceptibility to teicoplanin 30 µg/disc (TEC₃₀), vancomycin 30 µg/disc (VAN₃₀), chloramphenicol 30 µg/disc (C₃₀), tetracycline 30 µg/disc (TE₃₀), erythromycin 15 µg/disc (E₁₅), gentamycin 10 µg/disc (CN₁₀), kanamycin 30 µg/disc (K₃₀), streptomycin 10 µg/disc (S₁₀). Each strain was classified as sensitive (S) or intermediate (I) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). No resistant (R) strains were found

Discussion

It is well established that gastrointestinal microbiota is a key player in human and animal health (Wang et al. 2018a, b), and the use of probiotics represents an excellent approach to improve gut microbial balance and host health (Dimitroglou et al. 2011). One important criterion for probiotic selection is the capacity to minimize pathogens' growth by competitive exclusion or through the production of antimicrobial molecules (Verschuere et al. 2000; Dobson et al. 2012; Hai 2015; Banerjee and Ray 2017). The aquaculture sector growth is limited by frequent infection episodes (WHO 2014; Lafferty et al. 2015), but no specifically dedicated probiotics have so far been developed.

This study assumed that probiotics would be potentially more effective if originated from the gut of target animals and from the target pathogens ecological niche. Focus was on *Bacillus* species, which are natural inhabitants of the animal's gut, including fish (Barbosa et al. 2005; Hong et al. 2005; Tam et al. 2006; Zhou et al. 2014; Midhun et al. 2017; Guo et al. 2016) and are known producers of NACs capable of inhibiting pathogen's growth and proliferation (Abriouel et al. 2011; Caulier et al. 2019). In addition, different *Bacillus* spp. are commercially available probiotic products for human and animal use. Specifically, *B. subtilis*, *B. vallismortis*, *B. mojavensis*, *B. atropheus*, *B. amyloliquefaciens*, and *B. licheniformis* are included in the EFSA list of Qualified Presumption of Safety (QPS). However, no *Bacillus* probiotic is yet available for fish in the EU. To fulfil this market gap, we isolated several putative probiotic sporeforming *Bacillus* spp. from the gut of three marine aquaculture species, including gilthead seabream and European seabass (the most important fish species reared in the Mediterranean), and white seabream (a species with potential for aquaculture).

More than 50% of the *Bacillus* spp. isolates were active against at least one of the 14 tested bacterial fish pathogens, reinforcing their recognition, as producers of important bioactive metabolites (Caulier et al. 2019). Previous research reported that *Bacillus* spp. isolated from fish gut exhibited inhibitory activity against fish pathogens, including *Aeromonas* (Ramesh et al. 2015; Thankappan et al. 2015; Banerjee et al. 2016; Midhun et al. 2017; Ran et al. 2012; Guo et al. 2016; Meidong et al. 2018; Mukherjee et al. 2019; Serra et al. 2019; Askarian et al. 2012; Gao et al. 2017; Nandi et al. 2017; Kuebutornye et al. 2020) or *Vibrio* species (Chen et al. 2016; Serra et al. 2019; Askarian et al. 2012; Kuebutornye et al. 2020). The extent of the inhibitory actions observed in our study enlarges the potential role of fish-gut *Bacillus* community against furunculosis, vibriosis, photobacteriosis, tenacibaculosis and edwardsiellosis diseases, protecting the hosts against opportunistic bacteria.

Because *Bacillus* antimicrobial activity is usually a result of molecules produced and released to the surrounding environment (Abriouel et al. 2011; Caulier et al. 2019), the cell-free supernatants of each fish isolate were also tested for their antimicrobial activity. As expected, the anti-growth activity of *Bacillus* fish-gut isolates was mediated by extracellular NACs. In particular, the cell-free supernatants of strains FI314 and FI330 were highly effective in reducing the growth of all the tested fish pathogens. Unexpectedly, an increased growth of *A. salmonicida* and *V. harveyi* in the presence of FI359, FI376, and FI480 cell-free supernatants has been observed. A potential explanation of this evidence could be that cell-free supernatants might harbor substances that serve as nutrients for some bacteria (Moons et al. 2009; Hibbing et al. 2010). However, such hypothesis would need to be confirmed for our strains, under our experimental conditions. Another intriguing observation was the variation of results obtained when using two different antimicrobial assays for cell-free supernatant: only the extracellular NACs suppressing pathogen's growth for 24 h in liquid medium produced a strong inhibitory halo against the same pathogen onto agar medium. For example, the FI314 and FI330 extracellular NACs inhibited the growth of *S. sonnei*, in liquid medium without a visible inhibitory halo onto agar medium. Such differences may reflect technical limitations, since agar-based tests can be influenced by compound's diffusion into the agar, whereas the liquid-based assays allow full contact between the compounds and the target pathogenic strain. Thus, agar-based assays allow quick and less laborious screenings, but liquid-based assays are preferable when a quantitative analysis is to be done (Balouiri et al. 2016).

Bacterial biofilms are important key-parameters for bacterial proliferation and persistence, through the promotion of resistance and tolerance to external assaults, including antimicrobials (Flemming et al. 2016). The capacity of *Bacillus* spp. to regulate or inhibit pathogens biofilm formation has been already studied with the objective of controlling bacterial infections in medical and industrial fields (Nahar et al. 2018; Kalpana et al. 2012), including aquaculture (Hamza et al. 2016). Previous studies demonstrated *Bacillus* spp. potential in biofilm control of a broad range of aquatic pathogens, including *Aeromonas*, *Pseudomonas*, and *Vibrio* species (Vinoj et al. 2014; Chu et al. 2014; Hamza et al. 2018, 2016; Yatip et al. 2018; Kalpana et al. 2012). Accordingly, fish-gut isolates in the present study produced extracellular NACs capable of reducing biofilm formation of at least one of the tested pathogens, emphasizing the potential use of fish-gut *Bacillus* spp. in preventing the colonization and persistence of fish-pathogenic bacteria. Interestingly, and contrary to the anti-growth results, some fish-gut *Bacillus* extracellular NACs

could reduce *A. salmonicida* biofilm formation. This was the case of isolates FI359, FI376, and FI480. Similarly, FI359, FI376, and FI442 extracellular NACs also inhibited the biofilm production by *A. hydrophila*, and *S. sonnei* without interfering with their growth. These observations open the possibility of developing new prophylactic or therapeutic approaches, to deal with problematic fish pathogens and zoonotic agents, such as *A. salmonicida*. Although not directly inhibiting the growth of fish bacterial pathogens, *Bacillus* spp. might control their proliferation by reducing their defense mechanisms, such as biofilm (Grassi et al. 2017; Pletzer et al. 2016).

Another promising pathogen-controlling strategy is interfering with their communication or quorum-sensing, in a process called quorum-quenching (QQ) (Grandclement et al. 2016). Indeed, members of the *Bacillus* genus are known as prominent producers of QQ molecules, such as AHL-lactonases (*AiiA* and homologues) and one AHL-oxidoreductase (CYP102A1, P450BM-3) (Chowdhary et al. 2007; Chu et al. 2014; Dong et al. 2000; Tinh et al. 2013; Torabi Delshad et al. 2018; Vinoj et al. 2014; Zhou et al. 2016). The present results showed that FI314, FI330, and FI442 produce compounds capable of interfering with externally added 3-oxo-C6-HSL (in the case of *Chr. violaceum* CV026 biosensor) and with AHLs produced by the wild type *Chr. violaceum* CECT 494, namely N-(3-hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-C10-HSL), N-octanoyl-L-Homoserine lactone (C8-HSL) and other several minor AHLs (Morohoshi et al. 2008). The cell-free supernatant QQ-activity of these 3 promising *Bacillus* strains suggests an extracellular nature of the QQ molecules from FI314 and FI330. On the contrary, FI442 cell-free supernatant did not show any QQ activity, which indicates that its corresponding QQ molecules might possess an intracellular or cell-wall location, as reported in literature (Torabi Delshad et al. 2018; Cao et al. 2012; Chu et al. 2014; Pande et al. 2015; Romero et al. 2011; Zhou et al. 2018) or were degraded/inactivated under our experimental conditions.

Based on the results of the in vitro tests described in this study, the fish-gut isolates FI314, FI330, and FI442, identified as *B. subtilis*, were considered the most promising to be used as probiotics to incorporate in aquafeeds, or to become source of bioactive molecules able to antagonize important bacterial fish pathogens. Despite belonging to the same species (*B. subtilis*), the three selected isolates correspond to different strains. FI314 and FI330 were isolated from gilthead seabream (*Sparus aurata*), but from different digesta samples (different fish from different tanks) and FI442 was isolated from European seabass (*Dicentrarchus labrax*). Adding to the different origin and different colony morphologies, all three strains possess

different phenotypes (e.g., results on antimicrobial activities or sporulation efficiency), an indication of own genotypes.

Fish pathogens' infection routes are skin, gills, and gastrointestinal tract. In fact, some authors report the gastrointestinal tract as the main route of colonization in *Aeromonas* sp. (Lødemel et al. 2001; Ringø et al. 2004), *Vibrio* sp. (Oisson et al. 1996; Caruffo et al. 2015), and *Edwardsiella* sp. (Baldwin and Newton 1993), and imaging tools are being developed to visualize the exact dissemination of several pathogens inside the fish host (Bartkova et al. 2017; O'Toole et al. 2004). Thus, the selected *Bacillus* isolates and their NACs are expected to constitute a barrier against pathogens proliferation inside the animal's gut. This may be achieved indirectly through the regulation of inflammatory pathways by enhancing the host immune system (Canny and McCormick 2008) or directly either by killing the pathogens, by preventing pathogens' biofilm formation and subsequent gut colonizing, or by acting as quenching molecules shutting down pathogens communication or QS (Dobson et al. 2012; Wang et al. 2008; Abriouel et al. 2011; Sahoo et al. 2016). Importantly, NACs are directed to non-essential functions like biofilms or QS, and thus are less likely to induce resistance than common antibiotics (Sumi et al. 2014).

To confirm their potential probiotic application, the *Bacillus* fish-gut isolates were also evaluated for their capacity to produce spores. This feature is not only important from the production point of view, as the best sporeformers are the most attractive for high-yield fermentations, but also because spores allow bacteria to survive passage through the acidic stomach conditions, acting as a form of propagation inside the animal (Tam et al. 2006). All the isolated *Bacillus* strains tested showed a good sporulation efficiency, equivalent to the control laboratory strain *B. subtilis* 168. More importantly, all *Bacillus* isolates were susceptible to a series of antibiotic classes, including those demanded by EFSA as mandatory to comply with minimal safety requirements (Cabello et al. 2016; FEEDAP 2012) to be considered a probiotic.

The NACs produced by these *B. subtilis* strains, which are responsible for the observed anti-growth, anti-biofilm, and anti-QS activities, are currently being further studied for their isolation, identification, and full characterization. This is a challenging task, as *Bacillus* spp. produce a wide variety of antimicrobial molecules, from ribosomal peptides (RPs), volatile compounds, polyketides (PKs), and non-ribosomal peptides (NRPs) to hybrids between PKs and NRPs (Caulier et al. 2019). Interestingly, studies reporting the antimicrobial activity of *B. subtilis* against Gram-negative bacteria (the ones we target in this work) are scarce, when compared to the investigations on Gram-positive targets (Caulier et al. 2019; Olishavska et al.

2019). For example, seven compounds produced by *B. subtilis* sensu lato exhibited activity against gram-negative bacteria [Subtilosin A (Shelburne et al. 2007), Sonorensin (Chopra et al. 2014), CAMT2 (An et al. 2015), Subtulene A (Thasana et al. 2010), Lichenysin (Jenny et al. 1991), Lichenysin A (Yakimov et al. 1995), and Pumilacidin (Xiu et al. 2017)]. Among these compounds, only Subtilosin A and Subtulene A were isolated from *B. subtilis* sensu strictu, and their antimicrobial activity against fish pathogens is underexplored. Taking into account that 4–5% of the *B. subtilis* genome is dedicated to the production of antimicrobial compounds (Caulier et al. 2019) and the lack of studies on fish-gut associated *Bacillus* as producers of antimicrobial compounds (Soltani et al. 2019), the present investigation emphasizes the importance of described screening and bacterial collection, for the identification of new NACs with disease-preventive potential in aquaculture.

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Author Contributions Conceived and designed the experiments: RAS, AO-T, MJS, PE, CRS. Performed the fish trial: RAS, PP-F, PE. Performed the experiments and analyzed the data: RAS, CRS. Critically evaluated all the data and edited manuscript: RAS, AO-T, RJ, MJS, PE, CRS. Wrote the paper: RAS, CRS. All authors approved the final version of the manuscript.

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Data Availability 16S rRNA gene sequences of fish isolates described in this manuscript have been deposited in GenBank with the accession numbers provided in Table 2. Authors confirm that all relevant data are included in the article.

Declarations

Ethical Approval Animal experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and carried out in a registered installation (N16091.UDER) and were performed by trained scientists (following FELASA category C recommendations) in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Conflict of Interest RJ is an employee of Epicore Inc., a subsidiary of Archer Daniels Midland, that supported this research by completing a commercial licensing agreement for the potential utilization of the probiotic bacteria FI314, FI330, and FI442 identified in this research, in commercial products for aquaculture. The opinions expressed in the manuscript are those of the authors and do not necessarily reflect company policies. All other authors declare no competing interests.

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