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The marine symbiont *Pseudovibrio denitrificans,* is effective to control pathogenic *Vibrio* spp. in shrimp aquaculture

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ABSTRACT

In the search for probiotic bacteria from marine environments to control pathogenic *Vibrio* of cultivable marine species, this study focused on members of the genus *Pseudovibrio*. Based on its symbiotic features and antibacterial capacity, the genus *Pseudovibrio* could be used as probiotic to control vibriosis in shrimp culture. Forty-three isolates of *Pseudovibrio* were isolated from sponges *Aplysina gerardogreeni*, and *in vitro* competitive exclusion assays were performed against shrimp pathogenic *Vibrio* spp. (*Vibrio harveyi*, *Vibrio campbellii*, *Vibrio vulnificus* and two strains of *Vibrio parahaemolyticus*, one positive to PirA/PirB toxins). The most bioactive isolates (named Ps11, Ps17 and Ps18) against the pathogenic *Vibrio* spp. were validated by *in vivo* trials in the laboratory and in shrimp ponds. A significant increase in the survival of larvae and juveniles challenged. Additionally, the trial performed in PL3, which was naturally infected with *V. harveyi*, strongly indicates the *P. denitrificans* capabilities to compete and displace pathogenic *Vibrio* spp. present in shrimp postlarvae cultures enhancing their survival. Two isolates of *P. denitrificans*, coded as Ps17 and Ps18, were chosen due to the excellent results obtained *in vitro* and in the challenge tests and were assessed in experimental culture ponds of *Penaeus vannamei*. Ps17 and Ps18 were applied in the food once/day, during a complete culture cycle. Survival and production performances were significantly improved in ponds treated with Ps17. These results indicate the advantage of using *P. denitrificans* as probiotic for shrimp production.

1. Introduction

Some *Vibrio* spp. associated with shrimp mortality (Austin and Zhang, 2006; Goarant et al., 2006; Vanmaele et al., 2015; Soto-Rodriguez et al., 2015; Sathish Kumar et al., 2017), cause enormous economic losses in shrimp producing countries (Bell and Lightner, 1988; Tran et al., 2013; Dong et al., 2017a; Shinn et al., 2018). The most dramatic case is that of *Vibrio* causing acute hepatopancreatic necrosis disease (AHPND) for which several species have been found responsible (Tran et al., 2013; Liu et al., 2015; Dong et al., 2017b; Restrepo et al., 2018;) They carry a 70 kbp plasmid (pVA1) harboring the toxin genes PirA and PirB (Lee et al., 2015; Xiao et al., 2017; Liu et al., 2018). The poor efficiency of antibiotics and disinfectants to control these high virulent *Vibrio* spp. has led to a growing interest in the use of probiotics as epidemiological control tools. The use of probiotics is a common practice in current shrimp aquaculture. There are two main modes of action, namely competitive exclusion and immunomodulation (Lazado et al., 2011). Probiotics occupy and colonize the digestive tract, reducing the capacity of the pathogen to colonize (Chabrillón et al., 2005).

It is well known that probiotics can modify the gut microbiota in shrimp (Vargas-Albores et al., 2017). Through secretion of antibacterial substances, the probiotics are competing against pathogens, avoiding their adhesion to the intestine epithelium, competing for the necessary nutrients, and producing antitoxin effects (Martínez Cruz et al., 2012; Hai, 2015). There are numerous commercial probiotics, mainly based on bacterial strains *Lactobacillus* and *Bacillus* (Thammasorn et al., 2017; Zheng and Wang, 2017; Le and Yang, 2018). Regardless of their efficacy and possible benefits for shrimp, many of these bacteria are not typical of marine environments (Vargas-Albores et al., 2017), presenting problems to grow them in shrimp farming systems for *Penaeus vannamei* (Vargas-Albores et al., 2016), where they must compete against *Vibrio* spp., the latter being indigenous to the marine environment.

In the search for probiotic bacteria, typical of marine environments

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and capable of controlling pathogenic *Vibrio* spp. to shrimp, it is essential to explore other bacterial genera typical of marine environments. The symbiotic bacteria of marine organisms could be ideal candidates because they are adapted to marine conditions and can thrive in adverse environments (Esteves et al., 2017). It is well known that marine sponges harbor very diverse microbial communities, but only a small group of these symbiotic bacteria are cultivable (Taylor et al., 2007; Stewart, 2012; Esteves et al., 2016; Garate et al., 2017). The group bacteria that stands out the most as being cultivable is belonging to the genus *Pseudovibrio* (Esteves et al., 2013; Esteves et al., 2017; Nicacio et al., 2017). *Pseudovibrio* spp. have been isolated from several marine invertebrates, flatworms, sponges and tunicates (Sertan-De Guzman et al., 2007; Crowley et al., 2014; Zhang et al., 2016a. *Pseudovibrio* strains have the genomic potential to produce secondary metabolites and supply the host with cofactors through no-ribosomal peptide synthetase and polyketide synthase (NRPS/PKS) (Bondarev et al., 2013). Among several molecules with different biological activities, the molecules with the most dynamic antimicrobial activity are tropodithietic acid (TDA) (Harrington et al., 2014), alkaloids (He et al., 2017; Nicacio et al., 2017), and polyketides such as erythronolid (Esteves et al., 2017). Recently, it was reported that low concentrations of the TDA (0.5 μg mL^{-1}) exhibited activity against two coral pathogenic vibrios (Raina et al., 2016). Due to the production of new compounds and versatility of their metabolites (Bondarev et al., 2013; Naughton et al., 2017), the biotechnological potential of the genus *Pseudovibrio* has been pointed out by Crowley et al. (2014). Despite these properties, to date, there are no reports of the use of *Pseudovibrio* spp. in shrimp aquaculture to control emerging pathogenic *Vibrio* spp.

The aim of this study was to determine the probiotic capacity in shrimp culture of several isolates of *P. denitrificans* isolated from the marine sponge *Aplysina gerardogreeni* collected in the Marine Protected Area El Pelado (REMAPE), Santa Elena, Ecuador. Forty-three isolates of *Pseudovibrio* were obtained and *in vitro* competitive exclusion assays against shrimp pathogenic *Vibrio* spp. were carried out. The most bioactive strains were validated by *in vivo* trials in the laboratory and in shrimp ponds. In this article evidence is presented of the probiotic qualities of several isolates of *P. denitrificans*, highlighting their potential as useful tools to control emerging *Vibrio* spp. in shrimp aquaculture.

2. Materials and methods

2.1. Sample collection and bacterial isolation

A total of nine samples of the marine sponge *A. gerardogreeni*, were collected by SCUBA diving at four different rocky habitats at depths of 10–30 m from the REMAPE, located at the southern end of the tropical eastern Pacific, in the coastal waters of Santa Elena Province, Ecuador (01° 55, 9 ′S - 08° 47.2′ W). The sponge specimens were placed individually in Ziploc^{R} bags with seawater and transported to CENAIM laboratories. Endobionts and macroscopic epibionts were removed from sponge samples with scalpel and tweezers at the laboratory. Five grams of tissue taken from different parts of the organism were macerated and diluted in 45 mL sterile natural seawater (NSW). Subsequently, homogenized samples were serially diluted to 10^{-5} in NSW. One hundred (100) μL of dilutions 10^{-2} to 10^{-5} were streaked in triplicates onto Petri dishes containing BD DifcoTM Marine Agar 2216 (MA). Plates were incubated at 27 °C for two days. Afterward, different morphotypes were selected and purified by successive streaking in Petri dishes containing MA. *Pseudovibrio* strains were initially selected based on morphological and biochemical characteristics of the colonies previously described (Shieh et al., 2004; Sertan-De Guzman et al., 2007). Subsequently, the taxonomy of isolates exhibiting bioactivity was determined based on 16 rRNA gene nucleotide sequences. Pure cultures of *Pseudovibrio* were aliquoted in Luria-Bertani medium with NSW and 20% glycerol and preserved at −80 °C for subsequent trials.

2.2. Biochemical characterization of Pseudovibrio strains

Pseudovibrio isolates were characterized by biochemical tests including Gram stain, catalysis reaction, oxidase presence, amino acid (arginine, ornithine, and lysine) utilization, carbohydrate (sucrose, glucose, and mannose) utilization, use of citrate as a carbon source, the formation of diacetyl (Voges-Proskauer) and indole production. In addition, cell morphology was characterized, and sodium chloride (NaCl) requirement was evaluated by determining bacterial growth on LB broth cultures add 1% to 5% NaCl and LB broth (no additional NaCl).

2.3. In vitro assessment of anti-Vibrio bioactivity

The anti-*Vibrio* bioactivity of the *Pseudovibrio* isolates was evaluated against five shrimp pathogenic *Vibrio* species; *Vibrio harveyi* (strain E22), *Vibrio campbellii* (strain LM2013), *Vibrio vulnificus* (strain S2), *Vibrio parahaemolyticus* (ATCC 27969) and *Vibrio parahaemolyticus* (strain BA94C2, positive to PirA/PirB toxins) (Restrepo et al., 2016). The anti-*Vibrio* assessment was carried out with two methods, the spot inoculation on agar method described by Gao et al. (2017), employed to perform a fast screening, and the well agar diffusion method modified by Al Atya et al. (2015), to confirm the results.

2.3.1. Competitive exclusion test by spot inoculation on agar

This first screening was performed in order to identify *Pseudovibrio* isolates exerting a major anti-*Vibrio* potential. Individual fresh cultures of the five pathogenic *Vibrio* species were prepared on trypticase soy broth supplemented with 2% NaCl (wt/vol) (2% NaCl TSB) and adjusted to 2×10^{8} CFU mL^{-1} concentration correspondent to optical density ($O.D_{600}$) readings of 0.6 to 0.8. Two-hundred (200) μL of each bacterial suspension were plated by extension on plates containing MA. Each *Pseudovibrio* strain was inoculated as a spot (diameter ~2–3 mm) on the surface of a MA plate previously spread with pathogenic *Vibrio* suspensions. The plates were incubated at 28 °C for 72 h. The anti-*Vibrio* bioactivity was evaluated by the appearance of a growth inhibition zone around the spot. Only inhibition zones > 5 mm were considered positive reactions, as recommended by Zidour et al. (2017).

This second method of competitive exclusion was employed to obtain quantitative data using a standard concentration of the *Pseudovibrio* isolates. Preparation of the pathogenic *Vibrio* suspensions were identical to the spot inoculation method described before. Two hundred (200) μL of each bacterial suspension were spread on MA plates and 6 mm diameter wells were punched on the prepared agar plates with a sterile hollow glass rod. Individual colonies of *Pseudovibrio* isolates were inoculated into 10 mL LB medium prepared in NSW and incubated at 28 °C with shaking at 200 rpm for 24 h. The *Pseudovibrio* cultures were centrifuged (3000 *g*, 10 min, 4 °C), the supernatants discarded, and the cell pellets were resuspended in NSW. Dilutions were performed to obtain 1.1×10^{6} CFU mL^{-1} inocula. Fifty (50) μL of the *Pseudovibrio* suspensions were added to the wells. Each assay was performed in triplicate. The plates were incubated for 48 h and 72 h at 28 °C. Following incubation, antibacterial activity was assessed by measuring inhibition zones around the well containing the *Pseudovibrio*.

2.4. Amplification of 16S rRNA gene sequencing

The three most bioactive isolates (Ps11, ps17, and Ps18) were identified by molecular analysis. Bacterial DNA was extracted following the procedure outlined by Anand et al. (2006) with slight modifications. In summary, fresh individual colonies of each *Pseudovibrio* isolate were suspended in 500 μL of buffer solution (100 mM NaCl, 0.1 mM EDTA, 10 mM Tris-HCl pH 8.0) and 80 μL of 10% SDS. Buffered solutions were left for one hour in a water bath at 55 °C with the addition of 600 μL of phenol and centrifuged at 16000 *g* for 10 min. The supernatants were recovered and 100 μL of chloroform-isoamyl (24:1) was added. The mixtures were stirred and centrifuged at 16000 *g* for 10 min at 4 °C.

Two-hundred fifty (250) μL of 100% ethanol and 500 μL of ammonium acetate (5 M) were added to the mixtures. The solutions were stored at −20 °C overnight. Solutions were then centrifuged at 16000 *g* for 15 min at 4 °C and the supernatants were removed. The pellets were washed twice with 300 μL of 70% ethanol and allowed to dry for 2 h at 45 °C. Resulting DNA was finally resuspended in 50 μL of Milli-Q water. Amplification of the 16S rRNA gene was performed by PCR using the bacterial universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), specific for the domain bacteria (Lane, 1991). The PCR products of the 16 s rRNA strains Ps11, Ps17 and Ps18 were sent to Macrogen (Seoul, Korea) for sequencing. The sequences obtained were compared against public database using NCBI BLASTn, focusing on hits with an identity > 95%.

2.5. Safety evaluation of P. denitrificans on P. vannamei postlarvae survival

To rule out negative effects of *P. denitrificans* on shrimp larvae health, early zoea-1 stage shrimp were exposed to *Pseudovibrio* isolates Ps11, Ps17 and Ps18. The bioassay was performed in 6-well cell culture plates. Ten *P. vannamei* zoea-1 stage shrimp larvae were placed into each well. *Pseudovibrio* isolates were cultured as described previously. The *Pseudovibrio* cultures were centrifuged (3000 *g*, 10 min, 4 °C) and resulting bacterial pellets resuspended in sterile seawater and adjusted by optical densitometry (OD_{600} ~ 0.55) to equivalent colony concentrations of 1.5×10^8 CFU mL^{-1}. One-thousand (1000) μL of each *Pseudovibrio* suspension were directly applied to wells containing zoea-1 shrimp larvae (final dose of 1.5×10^7 CFU mL^{-1}). A control group containing zoea-1 stage shrimp larvae not exposed to *Pseudovibrio* was also included in the assay. Each treatment with 6 replicas. Survival was evaluated after 48 h of direct exposure. Survival of zoea-1 stage shrimp larvae in the control treatment was set to 100% and other treatments were normalized accordingly.

2.6. In vivo assay: effect of P. denitrificans on P. vannamei postlarvae exposed to Vibrio pathogens

2.6.1. Effect of P. denitrificans on the survival of P. vannamei postlarvae and juvenile by challenge test with V. campbellii and V. parahaemolyticus

First, the protective effect of *P. denitrificans* Ps11, Ps17 y Ps18 was assessed in healthy *P. vannamei* postlarvae (PL2), challenged with *V. campbellii* (LM2013). In the challenge test, 800 PLs were distributed in 16 glass flasks containing 2000 mL of filtered and UV sterilized sea water (Fifty PL2 per replicas). Continuous aeration was provided, and the temperature was set at 30.5 ± 0.4 °C. The PL2 were fed with commercial diet every four hours during all the bioassay. Three treatments (Ps11, Ps17 and Ps18) and a control (without *P. denitrificans* application) were applied. The *Pseudovibrio* isolates were prepared as described previously and applied in the water every twelve hours at a final concentration of 10^5 CFU mL^{-1}. Each treatment had four replicates. After five days the PLs were challenged with *V. campbellii* (10^6 CFU mL^{-1} of sea water). Water exchange (50%) was performed twenty-four hours post-exposure (hpe) and the survival of shrimp larvae quantified 48 hpe. In order to prepare bacterial inoculum, *V. campbellii* was plated on trypticase soy agar (2% NaCl TSA), and eight colonies were transferred to 1000 mL of 2% NaCl TSB and incubated for seven hours at 30 °C, with constant movement. Afterward, the culture was centrifuged (4000 *g*, 10 min, 25 °C), the supernatants discarded, and the cell pellets were resuspended in sterile saline solution (2% NaCl). *Vibrio* suspension was adjusted by optical densitometry at a density of 10^8 CFU mL^{-1}, and immediately inoculated to the concentration described before.

The protective effect of three isolates was also verified by a challenge test using healthy *P. vannamei* juveniles of weight 1.93 ± 0.66 g, with *V. parahaemolyticus* (BA94C2). In the challenge test, 160 shrimp juveniles were distributed in 16 glass flasks containing 2000 mL of filtered and UV sterilized sea water (Ten shrimp juveniles per experimental unit). Continuous aeration was provided, and the temperature was set at 31.2 ± 0.6 °C. The shrimp juveniles were fed with commercial diet every 8 h during all the bioassay. Three treatments (Ps11, Ps17, and Ps18) and a control (without *P. denitrificans* application) were applied. The *Pseudovibrio* isolates were prepared as described previously and applied in a single daily dose with the food at a concentration of (10^5 CFU g^{-1}). Each treatment had four replicates. After twelve days the shrimp juveniles were challenged with *V. parahaemolyticus* (10^6 CFU mL^{-1} of sea water). Water exchange (50%) was performed at 24 hpe and the survival was determined by counting the shrimp juveniles every four hours until 72 hpe. *V. parahaemolyticus* was activated in 2% NaCl TSB and the bacterial inoculum was prepared under the same conditions as *V. campbellii* inoculum. *V. parahaemolyticus* was immediately inoculated to the concentration described before.

2.6.2. Competitive exclusion effect of P. denitrificans against luminescent V. harveyi in naturally infected P. vannamei postlarvae

An *in vivo* trial was conducted to evaluate the ability of selected *Pseudovibrio* strains to exclude luminescent *V. harveyi* from infected *P. vannamei* shrimp postlarvae cultures. Three-day-old *P. vannamei* postlarvae (PL3) cultures infected with luminescent *V. harveyi* were obtained from a commercial shrimp hatchery and transferred to our bioassay settings at CENAIM. Postlarvae infection with luminescent *V. harveyi* at concentrations of 4.5×10^6 CFU g^{-1} was determined in our labs. One hundred PL3 shrimp postlarvae were randomly allocated to twelve 40-liter glass aquariums containing full strength filtered seawater 34.5 Practical Salinity Unit (PSU). The bioassay had 3 treatments (shrimp postlarvae treated with *Pseudovibrio* strains Ps11, Ps17, and Ps18) and one control group (untreated postlarvae). Each treatment had three replicates. *Pseudovibrio* suspensions were prepared similarly as described in previous sections. Three isolated of *Pseudovibrio* (Ps11, Ps17, and Ps18) were evaluated individually at a final concentration of 10^5 CFU mL^{-1}. After 48 of *Pseudovibrio* inoculation, 1 g of postlarvae samples from each aquarium was withdrawn. Postlarvae samples were washed and macerated in sterile saline solution (2% NaCl) (*w/v*), and serially diluted from 10^{-1} to 10^{-5} in sterile saline solution. Then, 100 μL of each dilution was plated on Petri dishes containing MA and Thiosulfate-citrate-bile salts-sucrose agar (TCBS modified 2% NaCl). The number of CFU g^{-1} was determined after 18–24 h of incubation at 28 °C.

2.7. Effect of Pseudovibrio application in P. vannamei grow-out ponds

2.7.1. Production of bacterial biomass

Two *P. denitrificans* isolates, Ps17 and Ps18 were grown by streaking individual Petri dishes and incubated at 26 °C for 48 h. Subsequently, individual colonies of *P. denitrificans* were transferred to LB medium (in NSW) and incubated for 48 h at 26 °C. After the incubation period, the bacterial culture was centrifuged at 4000 *g* for 10 min at 4 °C, and resulting microbial pellets resuspended in NSW and stored at room temperature (25 °C). The final concentration (CFU mL^{-1}) and bacterial viability were determined by plating the bacterial suspension in MA, as described previously.

2.7.2. Pond bioassay

Twelve 400 m^2 square earthen ponds of CENAIM's Experimental Station (Santa Elena Province) were randomly allocated to three *Pseudovibrio* application treatments; *Pseudovibrio* strain Ps17, *Pseudovibrio* strain Ps18 and no strain application (control group). Each treatment had four replicates ponds. A total of 3200 shrimp post-larvae PL12 stage were stocked in each pond (stocking density of 8 post-larvae per square meter). *P. denitrificans* isolates were applied in a single daily dose in the food at a concentration of (10^5 CFU g^{-1}) from the first day of culture throughout the whole production cycle that lasted 108 days. Each day the *Pseudovibrio* were incorporated into the commercial

pelleted feed (28% protein) and were immediately supplied to the assigned ponds. The commercial feed dose used was 3% of the average body weight of the shrimp, the latter being weekly verified. Environmental parameters such as temperature, dissolved O_2, and salinity, were monitored daily. Final shrimp survival (%), average weight (g), production yields (kg/ha) and feed conversion ratio (FCR) were the response variables of treatment effects evaluated at harvest. In addition, to confirm the presence of the *Pseudovibrio* in the treated shrimp, hepatopancreas, stomach, and intestine, were analyzed separately by classical microbiology. After the shrimp reached 2 g of body weight, 45 shrimp were randomly collected per treatment (15 per pond) every fifteen days during the whole production cycle. The shrimp were opened under strict conditions of asepsis to extract the three organs. Three pool were made per pond, consisting of 5 hepatopancreas, 5 stomachs and, 5 intestines. Each pool of organs was analyzed as an independent sample by triplicate. The presence of *Pseudovibrio* in the water and sediment of the ponds was also confirmed by microbiological analyzes. The microbial counts were expressed in CFU g^{-1} and CFU mL^{-1}, according to the sample analyzed (liquid or solid).

2.8. Statistical analysis

Data analysis for *in vitro* and *in vivo* assays was carried out using the statistical software SPSS (version 21). All data were analyzed using one-way ANOVA, after verification of the normality and variance homogeneity assumptions. When significant differences were detected a Dunnett's *post hoc* analysis was applied. The level of significance was set at ($P < .05$). The data expressed in percentages were transformed (using arcsin), and the assumptions were fulfilled before performing the statistical analysis.

3. Results

3.1. Isolated bacteria and morphological characterization

Forty-three isolates of *Pseudovibrio* spp. were isolated from several samples of *A. gerardogreeni* and classified according to morphological and biochemical features. Thirty-nine isolates exhibited morphological, physiological and biochemical characteristics similar to *P. denitrificans* (Shieh et al., 2004), while four isolates coded as Ps12, Ps35, Ps39, and Ps40, differed in three biochemical tests (Ornithine decarboxylase, lysine decarboxylase and citrate utilization) in respect to the original description of *P. denitrificans* (Table 1). In all 43 *Pseudovibrio* isolates, a sticky consistency was observed in the colonies, a characteristic not previously mentioned in the literature.

3.2. Anti-Vibrio bioactivity of Pseudovibrio isolates

3.2.1. Competitive exclusion test by spot inoculation on agar

Among the 43 isolates of *Pseudovibrio* spp. evaluated against pathogenic *Vibrio* spp., fifteen showed bioactivity against the five pathogenic *Vibrio* spp., twenty-three isolates exhibited bioactivity against at least one pathogen, while five isolates did not show any anti-*Vibrio* bioactivity. Most isolates of *Pseudovibrio* showed inhibitory zones of 8–15 mm in diameter (Fig. 1, Table 2). The isolates coded as Ps6, Ps11, Ps17, Ps18, and Ps37, showed larger anti-*Vibrio* bioactivity, forming exclusion zones larger than 20 mm against the five pathogens tested (Table 2). Three of the pathogenic *Vibrio* spp., *V. campbellii*, *V. vulnificus* and *V. parahaemolyticus* efficiently excluded by *P. denitrificans* (Fig. 1) were resistant to the antibiotic oxytetracycline.

3.2.2. Competitive exclusion by agar well diffusion test

The five isolates of *Pseudovibrio* (Ps6, Ps11, Ps17, Ps18, and Ps37) exhibiting higher anti-*Vibrio* bioactivity presented inhibition zones for all the *Vibrio* tested. However, the largest inhibition zones were exerted by the strains Ps11, Ps17, and Ps18 (Table 3). Therefore, only strains Ps11, Ps17, and Ps18 were further tested in the *in vivo* assays.

3.3. Molecular identification of the most bioactive Pseudovibrio strains against pathogenic Vibrio

Molecular analysis confirmed that *Pseudovibrio* isolates Ps11, Ps17, and Ps18 with largest anti-*Vibrio* bioactivity corresponded to *Pseudovibrio denitrificans* species previously described by Sertan-De Guzman et al. (2007) and Shieh et al. (2004). The partial 16S rDNA sequences of Ps11, Ps17 y Ps18 were submitted to GenBank and the accession number was assigned as Ps11MH201036, Ps17MH201037, Ps18MH201038. Followed by BLAST analysis, the partial 16S rRNA gene sequence of Ps11, Ps17 y Ps18 showed 100% similarity to *P. denitrificans*. Strains Ps11, Ps17 y Ps18 was deposited in the repository of the Centro Nacional de Acuicultura e Investigaciones Marinas (Ecuador).

3.4. Safety of P. denitrificans strains on shrimp early larvae P. vannamei

To rule out negative effects of *P. denitrificans* on shrimp larvae, early zoea-1 stage shrimp were exposed to isolates Ps11, Ps17 and Ps18. After 48 h of direct exposure, the survival of zoea-1 exposed to all the three *P. denitrificans* isolates was higher than 94%, not significantly different ($P < .05$) to the control treatment.

3.5. In-vivo trial with P. denitrificans

3.5.1. Effect of P. denitrificans on survival of P. vannamei postlarvae and juvenile by challenge test with V. campbellii and V. parahaemolyticus

In the first challenge, the survival of the postlarvae challenged with *V. campbellii* increased in the three groups treated with the presumptive probiotics Ps11, Ps17, and Ps18. Significant differences in survival were observed ($P < .05$) between the postlarvae treated with the isolates Ps11 and Ps17 (survival 56 ± 10% and 53 ± 9%) and the control group (survival 30 ± 8%) (Fig. 2A). In the second challenge, performed using juvenile shrimp challenged with *V. parahaemolyticus*, the survival in the control group was 17 ± 9%, while the survival (38 ± 10%, 45 ± 6% and 42 ± 12%) in the treatments was significantly higher ($P < .05$), for Ps11, Ps17 and Ps 18 respectively (Fig. 2B).

3.5.2. Beneficial effect of the Pseudovibrio excluding luminescent Vibrio in naturally infected P. vannamei larvae

The evaluated *Pseudovibrio* (Ps11, Ps17 and Ps18) efficiently displaced the luminescent *V. harveyi* in infected *P. vannamei* postlarvae (PL3). After 48 h *P. denitrificans* was established in the postlarvae bacterial population, reaching 50% of total shrimp postlarvae cultivable bacterial biomass (Fig. 3). Total *Vibrio* and *V. harveyi* were significantly reduced ($P < .05$) with respect to the control group. In this trial it was observed that, in addition, the survival of postlarvae exposed to *P. denitrificans* was 57 ± 17%, 79 ± 12%, and 69 ± 22%, for Ps11, Ps17 and Ps18, respectively, while the survival of the control was 49%, without significant difference ($P < .05$).

3.6. P. denitrificans application in the cultivation of P. vannamei: pond bioassay

Environmental parameters were kept within acceptable ranges for *P. vannamei* culture throughout the experiment. The average temperature was 26.5 °C, dissolved oxygen levels always remained above 3.5 mg L^{-1} and the salinity fluctuated between 34 and 45 g L^{-1}. The final survival for treatments Ps17, Ps18 and control was 79 ± 5%, 66 ± 7%, 63 ± 7%, respectively. Survival of treatment Ps17 was significantly higher ($P < .05$) as compared to treatment Ps18 and control group (Table 4). The production yield of *P. denitrificans* treated pond Ps17 was also significantly higher ($P < .05$) as compared to

Table 1
Characteristics of strains *Pseudovibrio* isolates.

Characteristics tested	Isolates exhibiting biochemical features of *P. denitrificans*. Ps:1,2,3,4,5,6,7,8,9,10,11,13,14,15,16,17,18,19,20,21,22,23,24,25,26,17,28,29,30,31,32,33,34,36,37,38,41,42,43.	Isolates exhibiting a difference in three biochemical tests in respect of *P. denitrificans*. Ps:12, 35, 39 y 40.	*Pseudovibrio denitrificans* strain DN34[Ta]
Colony characteristics	Convex colony, with an entire margin, big size, chewy appearance, whitish when young and turns brown on marine agar as the culture ages, nonluminescent	Convex colony, with an entire margin, big size, chewy appearance, translucent when young and turns in bone color on marine agar as the culture ages, nonluminescent	Circular, nonluminescent, with an entire margin, translucent.
Gram reaction	−	−	−
Cell shape	Rod, straight or curved	Rod, straight or curved	Rod, straight or curved
Motility	+	+	+
Range temperature for growth	25–31 °C	25–31 °C	30 °C
Range NaCl requirement for growth	3–4%	3–4%	3%
Fermentation of glucose	+	−	+
Fermentation of mannose	+	+	+
Fermentation of sucrose	+	+	+
Nitrate reduction	+	+	+
Oxidase	+	+	+
N-Acetyl-D-glucosamine	+	+	ND
Catalase	+	+	+
Gelatinase	+	+	+
Arginine dihydrolase	−	−	−
Ornithine decarboxylase	−	+	−
Lysine decarboxylase	−	+	−
Citrate utilization	−	+	−
H_2S production	−	−	−
Esculin hydrolysis	+	+	+
Indole production	+	+	+

[a] Data from Shieh et al., 2004.

treatment Ps18 and the control group. Feed conversion ratio among treatments was not different ($P < .05$), ranging between 1.0 and 1.3 (Table 4). Regarding the presence of *P. denitrificans* in the analyzed shrimp, on day 30, (10^3 CFU g^{-1}) were registered in the stomach and (10^4 CFU g^{-1}) in the digestive tract. The values increased to (10^5 CFU g^{-1}) in the digestive tract and (10^4 CFU g^{-1}) in the stomach on day 45 and remained in the same order of magnitude throughout the experiment. There was, however, no evidence of *P. denitrificans* colonies in the hepatopancreas analyzed. In the water and sediment of the treated ponds, the values fluctuated between (10^3 at 10^4 CFU g^{-1}) and (10^2 at 10^4 CFU mL^{-1}), for water and sediment, respectively.

4. Discussion

Commercial probiotics used in shrimp culture belong predominantly to the genera *Lactobacillus* and *Bacillus* (Hong et al., 2005; Martínez Cruz et al., 2012). Although some commercially used species are marine, the majority of commercial probiotic bacteria are of terrestrial origin, because they were initially formulated for other terrestrial animals (Lauzon et al., 2008). Although these formulated bacteria are of proven efficacy for the original target organisms, their efficiency can be compromised when the environmental conditions are very different, as in the case of marine shrimp cultures. The penaeid shrimp are subject to high salinity and temperature, which favor the growth of *Vibrio* spp. (Vezzulli et al., 2013; Zhang et al., 2016b). The optimum range of salinity and temperature to cultivate *P. vannamei*, is 20 to 35 ups and between 22 and 32 °C. Using metagenomics, Zhang et al. (2016b), shows that salinity modifies the microbiota in shrimp, finding that at higher salinity, *Vibrio* are dominant to the detriment of *Lactobacillus*, while Vezzulli et al. (2013), report that a temperature

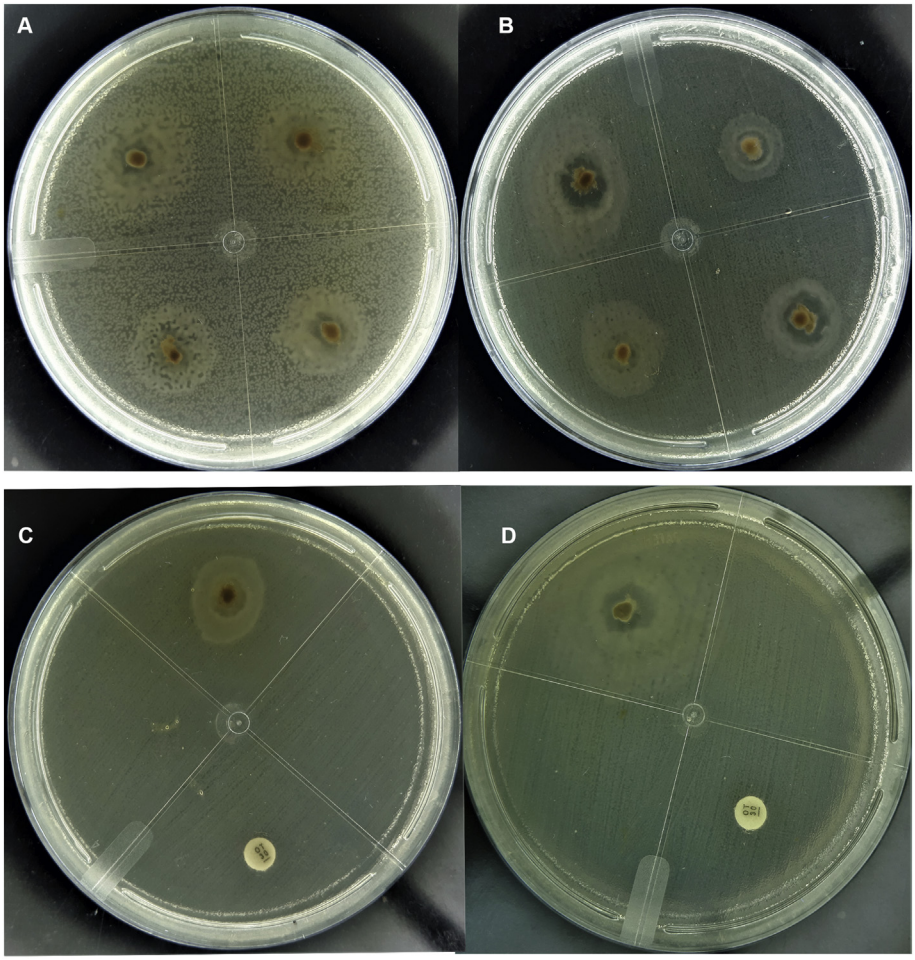

Fig. 1. Bioactivity of *Pseudovibrio* isolated, against pathogenic *Vibrio*. A) *Pseudovibrio* spp., front *V. harveyi* (E22). B) *Pseudovibrio* spp., front *Vibrio campbellii* (LM-2013). C) *Pseudovibrio* spp., front *V. vulnificus* (S2) and disc of oxytetracycline and D) *Pseudovibrio* spp., front *V. parahaemolyticus* (BA94C2) and a disc of oxytetracycline.

lower than 37 °C negatively affects the growth of the *Lactobacilli*.

Marine biodiscovery is a promising alternative, for isolating marine bacteria that function as effective probiotics for cultivable marine organisms. Marine invertebrates, particularly sponges, host a great bacterial diversity. Among the cultivable marine bacteria, the genus *Pseudovibrio* stands out, and in the last decades it has been the target of studies due to its versatility of producing bioactive molecules against Gram-negative and positive bacteria (O'Halloran et al., 2011; Penesyan et al., 2011; Bondarev et al., 2013; Naughton et al., 2017; Romano, 2018). In this study, we isolated several isolates of *P. denitrificans*, exhibiting probiotic qualities for shrimp, due to their strong anti-*Vibrio* bioactivity. These qualities were demonstrated in our trials, challenge test and in culture ponds.

In this study, bioactivity was found against *V. parahaemolyticus*, *V. campbellii*, *V. vulnificus* and *V. harveyi* and the highly virulent pathogenic *V. parahaemolyticus* strain BA94C2 positive to PirA/PirB, toxins associated to AHPND pathologies in shrimp cultures (FAO, 2013; Lai et al., 2015; Lee et al., 2015; Dong et al., 2017a; Zheng et al., 2018). To our knowledge, this is the first report analyzing the anti-*Vibrio* potential of *Pseudovibrio* against pathogenic *Vibrio* spp. in shrimp. The antibacterial activity of the genus *Pseudovibrio* has been mentioned in numerous studies (O'Halloran et al., 2011; Crowley et al., 2014; Raina et al., 2016; Naughton et al., 2017), against Gram-positive and Gram-negative bacteria, including *E. coli*, *Bacillus subtilis*, *Kluyveromyces marxianus*, *Salmonella enterica* serotype Typhimurium, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Flemer et al., 2012; Margassery et al., 2012). Recently, activity against two pathogenic *Vibrio* found in corals, *Vibrio coralliilyticus* and *Vibrio owensii* (Raina et al., 2016) was reported. The antibacterial activity of *Pseudovibrio* strains has been associated with its metabolome, sulfuric acid, tropodiethetic acid (TDA) (Penesyan et al., 2011; Crowley et al., 2014; Harrington et al., 2014). The TDA can kill or inhibit *Vibrio* spp. pathogens in fish larvae (Grotkjær et al., 2016). In addition, *Pseudovibrio* being a bacterium typical of marine environments (Versluis et al., 2018), the salinity favors their growth. Moreover, *Pseudovibrio* isolates had an optimal growth between 25 at 31 °C. These conditions favor its ability to compete against *Vibrio* spp.

A very important step to evaluate probiotic features is to rule out the innocuity of bacterial candidates. The results corroborate the innocuity of three *Pseudovibrio* isolates assessed in zoea-1 shrimp larvae. The microbiological analysis confirmed that the *Pseudovibrio* were associated with the zoea-1, without provoking adverse effects. Several *Pseudovibrio* spp. have been reported as symbionts of many marine benthic invertebrates, including shrimp (Romano, 2018). In addition, *Pseudovibrio* is well equipped to survive in adverse environments where nutrients may fluctuate, such as shallow seawater (Fukunaga et al., 2006) and are able to proliferate in ultra-oligotrophic sea waters (Schwedt et al., 2015). This metabolic versatility of the genus favors its association with their invertebrate hosts and are beneficial or at least neutral (Versluis et al., 2018; Alex and Antunes, 2018), since most of them are commonly associated with healthy animals. After the safety trials, the next step was to determine the effectiveness of *P. denitrificans* against pathogenic *Vibrio* spp. *in vivo* tests.

In order to verify the effectiveness of the *P. denitrificans* as probiotic,

Table 2
Antagonistic activity of different isolates of *Pseudovibrio* against five shrimp pathogenic *Vibrio* spp., by Spot Inoculation on Agar. Exclusion halo expressed in (mm) after incubation for 72 h.

Codes	*V. parahaemolyticus* (27969)	*V. campbellii* (LM2013)	*V. parahaemolyticus* (BA94C2)	*V. vulnificus* (S2)	*V. harveyi* (E22)	Spectrum
Ps-1	15	11	11	15	20	5/5
Ps-2	15	24	–	16	21	4/5
Ps-3	17	11	12	17	15	5/5
Ps-4	–	30	–	7	17	3/5
Ps-5	8	26	13	15	26	5/5
Ps-6	23	25	21	25	19	5/5*
Ps-7	8	18	11	15	18	4/5
Ps-8	–	–	–	–	–	0/5
Ps-9	–	21	10	13	18	4/5
Ps-10	5	8	13	10	15	3/5
Ps-11	23	22	21	20	25	5/5*
Ps-12	–	3	11	–	18	2/5
Ps-13	–	–	15	–	–	1/5
Ps-14	10	21	13	11	20	5/5
Ps-15	–	–	6	–	12	1/5
Ps-16	11	1	8	21	21	4/5
Ps-17	22	30	35	27	31	5/5*
Ps-18	32	35	40	21	35	5/5*
Ps-19	–	21	6	21	11	3/5
Ps-20	6	35	10	22	29	4/5
Ps-21	–	–	–	–	–	0/5
Ps-22	18	16	17	19	17	5/5
Ps-23	–	–	–	27	–	1/5
Ps-24	–	–	–	12	–	1/5
Ps-25	10	12	15	11	18	5/5
Ps-26	–	4	–	–	–	0/5
Ps-27	–	17	–	1	–	1/5
Ps-28	10	11	15	15	21	5/5
Ps-29	19	15	17	16	12	5/5
Ps-30	–	5	–	6	–	0/5
Ps-31	–	–	4	–	–	0/5
Ps-32	15	15	14	11	11	5/5
Ps-33	–	–	15	9	4	1/5
Ps-34	22	24	12	22	25	5/5
Ps-35	12	7	10	5	–	2/5
Ps-36	6	3	4	12	–	1/5
Ps-37	22	24	23	22	25	5/5*
Ps-38	6	13	4	11	–	2/5
Ps-39	–	–	–	13	–	1/5
Ps-40	–	–	–	16	–	1/5
Ps-41	15	14	14	15	11	5/5
Ps-42	5	–	13	–	–	1/5
Ps-43	3	14	4	11	–	2/5

(−) means non-bioactive and (*) means activity > 20 mm for all pathogens.

Table 3
Antagonistic activity of most active isolates of *Pseudovibrio* against pathogenic *Vibrio* spp. of shrimp, by Well Agar Diffusion Method. Exclusion halo expressed in (mm) after incubation for 72 h.

Codes	*V. parahaemolyticus* (27969)	*V. campbellii* (LM2013)	*V. parahaemolyticus* (BA94C2)	*V. vulnificus* (S2)	*V. harveyi* (E22)
Ps-6	12	9	11	13	10
Ps-11	14	12	10	14	11
Ps-17	13	15	12	13	12
Ps-18	14	14	13	12	13
Ps-37	10	11	10	12	9

two challenge tests were performed employing two highly virulent vibrios *V. campbellii* and *V. parahaemolyticus*. A significant increase in the survival of larvae and juveniles challenged. Additionally, the trial performed in PL3, which was naturally infected with *V. harveyi*, strongly indicates the *P. denitrificans* capabilities to compete and displace pathogenic *Vibrio* spp. present in shrimp postlarvae cultures enhancing their survival. This finding is very relevant, considering that bacteria of the *Vibrio* genus are quite adapted to the culture environment of marine organisms (Moss and Leamaster, 2000; Tzuc et al., 2014). There were no significant differences in the survival of shrimp larvae treated with (Ps11, Ps17 end Ps18) compared to the control group. The high mortality observed in the postlarvae in the control group was likely related to the high load of luminescent *V. harveyi* present. Luminescent *Vibrio* spp. have been associated with severe mortalities in shrimp culture, mainly larvicultures in shrimp hatcheries (Defoirdt et al., 2012; Zhou et al., 2012).

The pond trial confirmed the beneficial effects of *P. denitrificans* application, demonstrating its effectiveness on growing out production, increasing survival, average shrimp harvest weight, and yields. Best production performance was registered with *P. denitrificans* isolate Ps17 when compared to isolate Ps18. This result indicates that tested *P. denitrificans* isolates can exert different effects, and therefore caution should be exercised when extrapolating the beneficial effect of *P. denitrificans* strains. The variability in bioactivity obtained from different isolates indicates the need to start similar studies using several isolates. In our study, we employed nine samples of sponge, taken in different places, to increase the possibility of obtaining a wide genetic pool of bacteria. Similar results have been found in other bacteria. Thus, the strain (ILI) of *V. algynoliticus* functions as a probiotic for shrimp (Rodríguez et al., 2007), while other strains of the same species have been reported as pathogenic (Liu et al., 2004). In the same way, Sonnenschein et al. (2018), reports different toxicity against microalgae

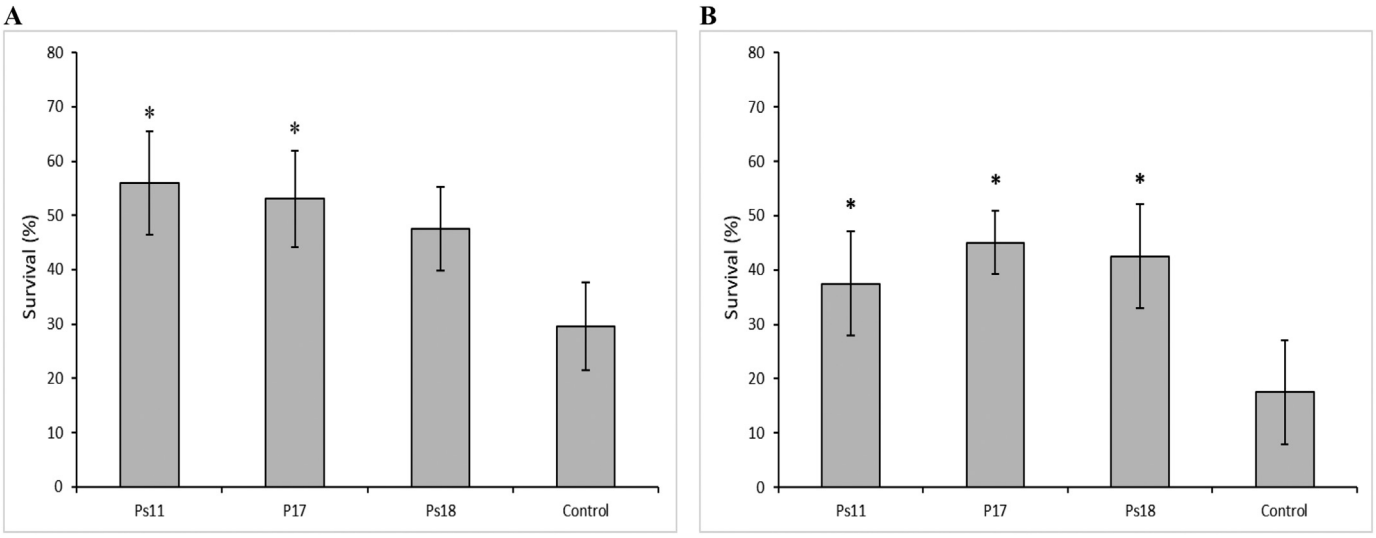

Fig. 2. A) Effect of the three *Pseudovibrio* isolates on the survival of the postlarvae *P. vannamei*, challenged with *V. campbellii*, initially treated for five days with the *Pseudovibrio*, survival (%) 72 hpe. B) Effect of *Pseudovibrio* on the survival of juveniles of *P. vannamei*, challenged with *V. parahaemolyticus*, initially treated for ten days with *Pseudovibrio*, survival (%) 72 hpe. The error bar represents the S.D. of the mean ($n = 4$). The asterisk (*) represents significantly different from the control ($P < .05$, Dunnett's).

of different strains of roseobacters.

5. Conclusion

Marine biodiscovery is a promising alternative for isolating marine bacteria, working as effective probiotics for cultured marine organisms. To identify, *Pseudovibrio* spp. highly bioactive against pathogenic *Vibrio* spp. of shrimp, several samples were taken from different morphotypes of *A. gerardogreeni* sponge, at several places of the Marine Protected Area El Pelado (REMAPE). Forty-three isolates were obtained, between them, three isolates coded as Ps11, Ps17 and Ps18 showed the highest bioactivity against *Vibrio* spp. highly pathogenic of shrimp. The anti-*Vibrio* bioactivity demonstrated by *in vitro* assays was confirmed by the beneficial effect observed in the challenge tests, the naturally infected larvae, and the shrimp culture ponds. The results of this study indicate that *Pseudovibrio* can be used as a biological control for *Vibrio* spp. in shrimp culture. Further study is needed to evaluate the practical concentrations on a commercial scale.

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Author contributions

J.R. and C.D-B., design the experiment; C.D-B and B·C-A. collected sponges and C.T performed molecular identification; C.D-B, V.A, M.B, and B·C-A. performed the experiments and bioassays; C.D-B, J.R, V.A, and M.B wrote the paper; J.R and S·S corrected and edited the manuscript before submission. All the authors have contributed to and

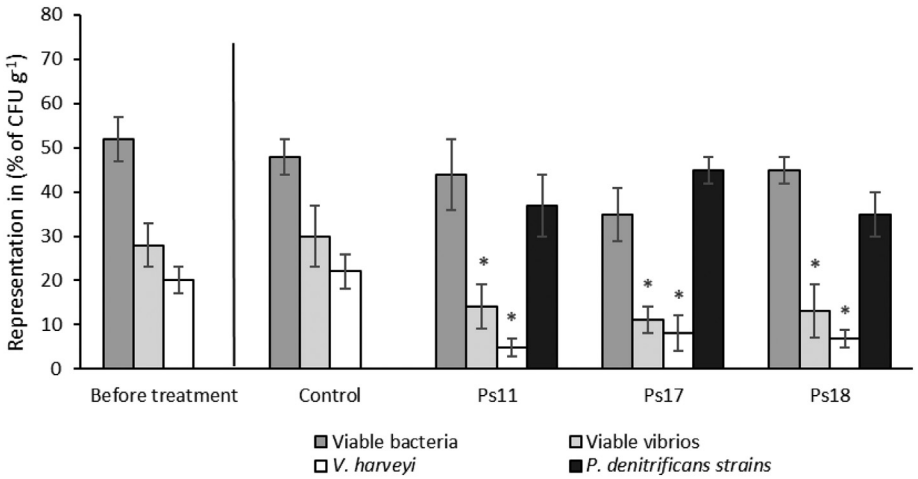

Fig. 3. Efficacy of *P. denitrificans* strains to colonize and displace total *Vibrio* and luminescent *Vibrio* in shrimp after 48 h of *P. denitrificans* application. The error bar represents the S.D. of the mean ($n = 3$). Asterisk (*) represent significantly different from control ($P < .05$, Dunnett's).

Table 4
The beneficial effect of *Pseudovibrio* strains on shrimp culture *P. vannamei* during bioassay of 108 days (production cycle duration). Results are presented as mean ± SD (n = 4). Different lowercase letters indicate significant differences ($P < .05$).

Treatments	Stocking density (shrimp/m^2)	SRG (% day^{-1})	Average weight (g)	Survival (%)	Yield (kg/ha)	FCR
Ps-17	8	7.7 ± 0.2 a	10.2 ± 2.0 a	79.2 ± 5.3 a	899.6 ± 59.5 a	0.99 ± 0.16 a
Ps-18	8	7.7 ± 0.3 a	10.8 ± 3.4 a	66.3 ± 6.8 b	753.4 ± 77.2 b	1.14 ± 0.28 a
Control	8	7.6 ± 0.3 a	9.4 ± 2.4 a	63.0 ± 6.7 b	715.9 ± 76.4 b	1.35 ± 0.32 a

approved the final manuscript.

Conflicts of interest

All authors declare no conflict of interest.

References

Al Atya, A.K., Drider-Hadiouche, K., Ravallec, R., Silvain, A., Vachee, A., Drider, D., 2015. Probiotic potential of *Enterococcus faecalis* strains isolated from meconium. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00227.

Alex, A., Antunes, A., 2018. Genus-wide comparison of *Pseudovibrio* bacterial genomes reveal diverse adaptations to different marine invertebrate hosts. PLoS One 13, 1–25. https://doi.org/10.1371/journal.pone.0194368.

Anand, T.P., Bhat, A.W., Shouche, Y.S., Roy, U., Siddharth, J., Sarma, S.P., 2006. Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. Microbiol. Res. 161, 252–262. https://doi.org/10.1016/j.micres.2005.09.002.

Austin, B., Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol. 43, 119–124. https://doi.org/10.1111/j.1472-765X.2006.01989.x.

Bell, T.A., Lightner, D.V., 1988. A Handbook of Normal Penaeid Shrimp Histology. World Aquaculture Society, Baton Rouge (No. 595.3843 B4).

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., Schulz-Vogt, H.N., 2013. The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. Environ. Microbiol. 15, 2095–2113. https://doi.org/10.1111/1462-2920.12123.

Chabrillón, M., Rico, R.M., Arijo, S., Díaz-Rosales, P., Balebona, M.C., Moriñigo, M.A., 2005. Interactions of microorganisms isolated from gilthead sea bream, *Sparus aurata* L., on *Vibrio harveyi*, a pathogen of farmed Senegalese sole, *Solea senegalensis* (Kaup). J. Fish Dis. 28, 531–537. https://doi.org/10.1111/j.1365-2761.2005.00657.x.

Crowley, S.P., O'Gara, F., O'Sullivan, O., Cotter, P.D., Dobson, A.D.W., 2014. Marine *Pseudovibrio sp.* as a novel source of antimicrobials. Marine Drugs 12, 5916–5929. https://doi.org/10.3390/md12125916.

Defoirdt, T., Benneche, T., Brackman, G., Coenye, T., Sorgeloos, P., Scheie, A.A., 2012. A quorum sensing-disrupting brominated thiophenone with a promising therapeutic potential to treat luminescent vibriosis. PLoS One 7, 1–7. https://doi.org/10.1371/journal.pone.0041788.

Dong, X., Bi, D., Wang, H., Zou, P., Xie, G., Wan, X., Yang, Q., Zhu, Y., Chen, M., Guo, C., Liu, Z., Wang, W., Huang, J., 2017a. pirABvp-Bearing *Vibrio parahaemolyticus* and *Vibrio campbellii* pathogens isolated from the Same AHPND-affected pond possess highly similar pathogenic plasmids. Front. Microbiol. 8, 1–9. https://doi.org/10.3389/fmicb.2017.01859.

Dong, X., Wang, H., Xie, G., Zou, P., Guo, C., Liang, Y., Huang, J., 2017b. An isolate of *Vibrio campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease. Emerging Microbes and Infections 6, e2–e3. https://doi.org/10.1038/emi.2016.131.

Esteves, A.I.S., Hardoim, C.C.P., Xavier, J.R., Gonçalves, J.M.S., Costa, R., 2013. Molecular richness and biotechnological potential of bacteria cultured from Irciniidae sponges in the north-east Atlantic. FEMS Microbiol. Ecol. 85, 519–536. https://doi.org/10.1111/1574-6941.12140.

Esteves, A.I.S., Amer, N., Nguyen, M., Thomas, T., 2016. Sample processing impacts the viability and cultivability of the sponge microbiome. Front. Microbiol. 7, 1–17. https://doi.org/10.3389/fmicb.2016.00499.

Esteves, A.I.S., Cullen, A., Thomas, T., 2017. Competitive interactions between sponge-associated bacteria. FEMS Microbiol. Ecol. 93, 1–8. https://doi.org/10.1093/femsec/fix008.

FAO, 2013. Report of the FAO/MARD Technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp (under TCP/VIE/3304). In: FAO Fisheries and Aquaculture Report.

Flemer, B., Kennedy, J., Margassery, L.M., Morrissey, J.P., O'Gara, F., Dobson, A.D.W., 2012. Diversity and antimicrobial activities of microbes from two Irish marine sponges, *Suberites carnosus* and *Leucosolenia sp.* J. Appl. Microbiol. 112, 289–301. https://doi.org/10.1111/j.1365-2672.2011.05211.x.

Fukunaga, Y., Kurahashi, M., Tanaka, K., Yanagi, K., Yokota, A., Harayama, S., 2006. Pseudovibrio ascidiaceicola sp. nov., isolated from ascidians (sea squirts). Int. J. Syst. Evol. Microbiol. 56, 343–347. https://doi.org/10.1099/ijs.0.63879-0.

Gao, X.-Y., Liu, Y., Miao, L.-L., Li, E.-W., Hou, T.-T., Liu, Z.-P., 2017. Mechanism of anti-*Vibrio* activity of marine probiotic strain *Bacillus pumilus* H2, and characterization of the active substance. AMB Express 7, 23. https://doi.org/10.1186/s13568-017-0323-3.

Garate, L., Sureda, J., Agell, G., Uriz, M.J., 2017. Endosymbiotic calcifying bacteria across sponge species and oceans. Sci. Rep. 7. https://doi.org/10.1038/srep43674.

Goarant, C., Reynaud, Y., Ansquer, D., Decker, S. de, Saulnier, D., Roux, F. le, 2006. Molecular epidemiology of *Vibrio nigripulchritudo*, a pathogen of cultured penaeid shrimp (*Litopenaeus stylirostris*) in New Caledonia. Syst. Appl. Microbiol. 29, 570–580. https://doi.org/10.1016/j.syapm.2005.12.005.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P., Dourala, N., Nielsen, K.F., Gram, L., 2016. Isolation of TDA-producing *Phaeobacter* strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in *Artemia* cultures. Syst. Appl. Microbiol. 39, 180–188. https://doi.org/10.1016/j.syapm.2016.01.005.

Hai, N.V., 2015. The use of probiotics in aquaculture. J. Appl. Microbiol. 119, 917–935. https://doi.org/10.1111/jam.12886.

Harrington, C., Reen, F.J., Mooij, M.J., Stewart, F.A., Chabot, J.B., Guerra, A.F., Glöckner, F.O., Nielsen, K.F., Gram, L., Dobson, A.D.W., O'Gara, F., 2014. Characterisation of non-autoinducing tropodithietic acid (TDA) production from marine sponge *Pseudovibrio* species. Marine Drugs 12, 5960–5978. https://doi.org/10.3390/md12125960.

He, W., Rahimnejad, S., Wang, L., Song, K., Lu, K., Zhang, C., Pezzani, R., Vitalini, S., Iriti, M., Cooper, D., Doucet, L., Pratt, M., Kim, J.D., Nhut, T.M., Hai, T.N., Ra, C.S., Ozdemir, N., Ozgen, Y., Kiralan, M., Bayrak, A., Arslan, N., Ramadan, M.F., Sarikurkcu, C., Zengin, G., Oskay, M., Uysal, S., Ceylan, R., Aktumsek, A., Rodrigues, D., Alves, C., Horta, A., Pinteus, S., Silva, J., Culioli, G., Thomas, O.P., Pedrosa, R., Mosmann, T., Bassolé, I.H.N., Juliani, H.R., Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., De Feo, V., Muñoz, M., Cedeño, R., Rodríguez, J., Van Der Knaap, W.P.W., Mialhe, E., Bachère, E., Jose, S., Mohandas, A., Philip, R., Bright Singh, I.S., Hyldgaard, M., Mygind, T., Meyer, R.L., Abdullah, E., Idris, A., Saparon, A., Yang, Q., Scheie, A.A., Benneche, T., Defoirdt, T., 2017. Antitumor and antimicrobial potential of bromoditerpenes isolated from the Red Alga, *Sphaerococcus coronopifolius*. Pharmaceuticals 70, 1451–1474. https://doi.org/10.1016/0022-1759(83)90303-4.

Hong, H.A., Le, H.D., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29, 813–835. https://doi.org/10.1016/j.femsre.2004.12.001.

Lai, H., Hann, T., Ando, M., Lee, C., Chen, I., Chiang, Y., Takeyama, H., Hamaguchi, H., Lo, C., Aoki, T., Wang, H., 2015. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish and Shellfish Immunology 47, 1006–1014. https://doi.org/10.1016/j.fsi.2015.11.008.

Lane, D.J., 1991. 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. John Wiley & Sons 115–175.

Lauzon, H.L., Gudmundsdottir, S., Pedersen, M.H., Budde, B.B., Gudmundsdottir, B.K., 2008. Isolation of putative probionts from cod rearing environment. Vet. Microbiol. 132, 328–339. https://doi.org/10.1016/j.vetmic.2008.05.014.

Lazado, C.C., Caipang, C.M.A., Brinchmann, M.F., Kiron, V., 2011. *In vitro* adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. Vet. Microbiol. 148, 252–259. https://doi.org/10.1016/j.vetmic.2010.08.024.

Le, B., Yang, S.H., 2018. Probiotic potential of novel *Lactobacillus* strains isolated from salted-fermented shrimp as antagonists for *Vibrio parahaemolyticus*. J. Microbiol. 56, 138–144. https://doi.org/10.1007/s12275-018-7407-x.

Lee, C., Chen, I., Yang, Y., Ko, T., Huang, Y., Huang, J., Huang, M., 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. 112. pp. 1–6. https://doi.org/10.1073/pnas.1503129112.

Liu, C.H., Cheng, W., Hsu, J.P., Chen, J.C., 2004. *Vibrio alginolyticus* infection in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing. Dis. Aquat. Org. 61, 169–174. https://doi.org/10.3354/dao061169.

Liu, L., Xiao, J., Xia, X., Pan, Y., Yan, S., Wang, Y., 2015. Draft genome sequence of *Vibrio owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. Genome announcements 3, 3354. https://doi.org/10.1128/genomeA.01395-15.

Liu, L., Xiao, J., Zhang, M., Zhu, W., Xia, X., Dai, X., Pan, Y., Yan, S., Wang, Y., 2018. A *Vibrio owensii* strain as the causative agent of AHPND in cultured shrimp, *Litopenaeus vannamei*. J. Invertebr. Pathol. https://doi.org/10.1016/j.jip.2018.02.005.

Margassery, L.M., Kennedy, J., O'Gara, F., Dobson, A.D., Morrissey, J.P., 2012. Diversity and antibacterial activity of bacteria isolated from the coastal marine sponges *Amphilectus fucorum* and *Eurypon major*. Lett. Appl. Microbiol. 55, 2–8. https://doi.org/10.1111/j.1472-765X.2012.03256.x.

Martínez Cruz, P., Ibáñez, A.L., Monroy Hermosillo, O.A., Ramírez Saad, H.C., 2012. Use of probiotics in aquaculture. ISRN Microbiology 2012, 1–13. https://doi.org/10.5402/2012/916845.

Moss, S.M., Leamaster, R., 2000. Relative abundance and species composition of gram-negative, aerobic bacteria associated with the gut of juvenile white shrimp *Litopenaeus vannamei* reared in oligotrophic well water and eutrophic pond water. J. World Aquacult. Soc. 31 (2), 255–263. https://doi.org/10.1111/j.1749-7345.2000.tb00361.x.

Naughton, L.M., Romano, S., O'Gara, F., Dobson, A.D.W., 2017. Identification of secondary metabolite gene clusters in the *Pseudovibrio* Genus reveals encouraging biosynthetic potential toward the production of novel bioactive compounds. Front.

Microbiol. 8, 1–15. https://doi.org/10.3389/fmicb.2017.01494.
Nicacio, K.J., Ióca, L.P., Fróes, A.M., Leomil, L., Appolinario, L.R., Thompson, C.C., Thompson, F.L., Ferreira, A.G., Williams, D.E., Andersen, R.J., Eustaquio, A.S., Berlinck, R.G.S., 2017. Cultures of the marine bacterium *Pseudovibrio denitrificans* Ab134 produce bromotyrosine-derived alkaloids previously only isolated from marine sponges. J. Nat. Prod. 80, 235–240. https://doi.org/10.1021/acs.jnatprod.6b00838.
O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O'Gara, F., Dobson, A.D.W., 2011. Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. J. Appl. Microbiol. 110, 1495–1508. https://doi.org/10.1111/j.1365-2672.2011.05008.x.
Penesyan, A., Tebben, J., Lee, M., Thomas, T., Kjelleberg, S., Harder, T., Egan, S., 2011. Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio sp*. D323 and related sponge-associated bacteria. Marine Drugs 9, 1391–1402. https://doi.org/10.3390/md9081391.
Raina, J.-B., Tapiolas, D., Motti, C.A., Foret, S., Seemann, T., Tebben, J., Willis, B.L., Bourne, D.G., 2016. Isolation of an antimicrobial compound produced by bacteria associated with reef-building corals. PeerJ 4, e2275. https://doi.org/10.7717/peerj.2275.
Restrepo, L., Bayot, B., Betancourt, I., Pinzón, A., 2016. Draft genome sequence of pathogenic bacteria *Vibrio parahaemolyticus* strain Ba94C2, associated with acute hepatopancreatic necrosis disease isolate from South America. Genomics Data 9, 143–144. https://doi.org/10.1016/j.gdata.2016.08.008.
Restrepo, L., Bonny, B., Arciniegas, S., Leandro, B., Irma, B., Panc, F., Re, A., 2018. PirVP Genes Causing AHPND Identified in a New *Vibrio* Species (*Vibrio punensis*) within the Commensal Orientalis Clade. pp. 1–14. https://doi.org/10.1038/s41598-018-30903-x.
Rodríguez, J., Espinosa, Y., Echeverría, F., Cárdenas, G., Román, R., Stern, S., 2007. Exposure to probiotics and β-1,3/1,6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to White Spot Syndrome Virus challenge and pond culture. Aquaculture 273, 405–415. https://doi.org/10.1016/j.aquaculture.2007.10.042.
Romano, S., 2018. Ecology and biotechnological potential of bacteria belonging to the genus *Pseudovibrio*. Appl. Environ. Microbiol. 84. https://doi.org/10.1128/AEM.02516-17.
Sathish Kumar, T., Vidya, R., Kumar, S., Alavandi, S.V., Vijayan, K.K., 2017. Zoea-2 syndrome of *Penaeus vannamei* in shrimp hatcheries. Aquaculture 479, 759–767. https://doi.org/10.1016/j.aquaculture.2017.07.022.
Schwedt, A., Seidel, M., Dittmar, T., Simon, M., Bondarev, V., Romano, S., Lavik, G., Schulz-Vogt, H.N., 2015. Substrate use of *Pseudovibrio sp*. growing in ultra-oligotrophic seawater. PLoS One 10, 1–16. https://doi.org/10.1371/journal.pone.0121675.
Sertan-De Guzman, A.A., Predicala, R.Z., Bernardo, E.B., Neilan, B.A., Elardo, S.P., Mangalindan, G.C., Tasdemir, D., Ireland, C.M., Barraquio, W.L., Concepcion, G.P., 2007. *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. FEMS Microbiol. Lett. 277, 188–196. https://doi.org/10.1111/j.1574-6968.2007.00950.x.
Shieh, W.Y., Lin, Y.T., Jean, W.D., 2004. *Pseudovibrio denitrificans* gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. Int. J. Syst. Evol. Microbiol. 54, 2307–2312. https://doi.org/10.1099/ijs.0.63107-0.
Shinn, A.P., Pratoomyot, J., Griffiths, D., Trong, T.Q., Vu, N.T., Jiravanichpaisal, P., Briggs, M., 2018. Asian shrimp production and the economic costs of disease. Asian Fisheries Science S 31, 29–58.
Sonnenschein, E.C., Phippen, C.B.W., Bentzon-Tilia, M., Rasmussen, S.A., Nielsen, K.F., Gram, L., 2018. Phylogenetic distribution of roseobacticides in the Roseobacter group and their effect on microalgae. Environ. Microbiol. Rep. 10, 383–393. https://doi.org/10.1111/1758-2229.12649.
Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., Morales-Covarrubias, M.S., 2015. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. Appl. Environ. Microbiol. 81, 1689–1699. https://doi.org/10.1128/AEM.03610-14.
Stewart, E.J., 2012. Growing unculturable bacteria. J. Bacteriol. 194, 4151–4160. https://doi.org/10.1128/JB.00345-12.
Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol. Mol. Biol. Rev. 71, 295–347. https://doi.org/10.1128/MMBR.00040-06.
Thammasorn, T., Jitrakorn, S., Charoonnart, P., Sirimanakul, S., Rattanarojpong, T., Chaturongakul, S., Saksmerprome, V., 2017. Probiotic bacteria (*Lactobacillus plantarum*) expressing specific double-stranded RNA and its potential for controlling shrimp viral and bacterial diseases. Aquac. Int. 25, 1679–1692. https://doi.org/10.1007/s10499-017-0144-z.
Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Org. 105, 45–55. https://doi.org/10.3354/dao02621.
Tzuc, J.T., Escalante, D.R., Herrera, R.R., Cortés, G.G., Leticia, M., Ortiz, A., 2014. Microbiota from *Litopenaeus vannamei*: Digestive Tract Microbial Community of Pacific White Shrimp (*Litopenaeus vannamei*). pp. 1–10.
Vanmaele, S., Defoirdt, T., Cleenwerck, I., De Vos, P., Bossier, P., 2015. Characterization of the virulence of Harveyi clade vibrios isolated from a shrimp hatchery *in vitro* and *in vivo*, in a brine shrimp (*Artemia franciscana*) model system. Aquaculture 435, 28–32. https://doi.org/10.1016/j.aquaculture.2014.09.015.
Vargas-Albores, F., Martínez-Porchas, M., Arvayo, M.A., Villalpando-Canchola, E., Gollas-Galván, T., Porchas-Cornejo, M.A., 2016. Immunophysiological response of pacific white shrimp exposed to a probiotic mixture of proteobacteria and firmicutes in farm conditions. N. Am. J. Aquac. 78, 193–202. https://doi.org/10.1080/15222055.2016.1167797.
Vargas-Albores, F., Porchas-Cornejo, M.A., Martínez-Porchas, M., Villalpando-Canchola, E., Gollas-Galván, T., Martínez-Córdova, L.R., 2017. Bacterial biota of shrimp intestine is significantly modified by the use of a probiotic mixture: a high throughput sequencing approach. Helgol. Mar. Res. 71. https://doi.org/10.1186/s10152-017-0485-z.
Versluis, D., Nijsse, B., Naim, M.A., Koehorst, J.J., Wiese, J., Imhoff, J.F., Schaap, P.J., van Passel, M.W.J., Smidt, H., Sipkema, D., 2018. Comparative genomics highlights symbiotic capacities and high metabolic flexibility of the marine genus *Pseudovibrio*. Genome Biology and Evolution 10, 125–142. https://doi.org/10.1093/gbe/evx271.
Vezzulli, L., Colwell, R.R., Pruzzo, C., 2013. Ocean warming and spread of pathogenic Vibrios in the aquatic environment. Microb. Ecol. 65, 817–825. https://doi.org/10.1007/s00248-012-0163-2.
Xiao, J., Liu, L., Ke, Y., Li, X., Liu, Y., Pan, Y., Yan, S., Wang, Y., 2017. Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various *Vibrio* species. Sci. Rep. 7, 1–11. https://doi.org/10.1038/srep42177.
Zhang, Y., Li, Q., Tian, R., Lai, Q., Xu, Y., 2016a. *Pseudovibrio stylochi* sp. Nov., isolated from a marine flatworm. Int. J. Syst. Evol. Microbiol. 66, 2025–2029. https://doi.org/10.1099/ijsem.0.000984.
Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W.T., Du, Z., Li, E., 2016b. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72–80. https://doi.org/10.1016/j.aquaculture.2015.12.014.
Zheng, C.N., Wang, W., 2017. Effects of *Lactobacillus pentosus* on the growth performance, digestive enzyme and disease resistance of white shrimp, *Litopenaeus vannamei* (Boone, 1931). Aquac. Res. 48, 2767–2777. https://doi.org/10.1111/are.13110.
Zheng, Z., Aweya, J.J., Wang, F., Yao, D., Lun, J., Li, S., Ma, H., Zhang, Y., 2018. Acute hepatopancreatic necrosis disease (AHPND) related microRNAs in *Litopenaeus vannamei* infected with AHPND-causing strain of *Vibrio parahemolyticus*. BMC Genomics 19, 1–11. https://doi.org/10.1186/s12864-018-4728-4.
Zhou, J., Fang, W., Yang, X., Zhou, S., Hu, L., Li, X., Qi, X., Su, H., Xie, L., 2012. A nonluminescent and highly virulent *Vibrio harveyi* strain is associated with "bacterial white tail disease" of *Litopenaeus vannamei* shrimp. PLoS One 7, 19–22. https://doi.org/10.1371/journal.pone.0029961.
Zidour, M., Chevalier, M., Belguesmia, Y., Cudennec, B., Grard, T., Drider, D., Souissi, S., Flahaut, C., 2017. Isolation and characterization of bacteria colonizing *Acartia tonsa* copepod eggs and displaying antagonist effects against *Vibrio anguillarum*, *Vibrio alginolyticus* and other pathogenic strains. Front. Microbiol. 8, 1919. https://doi.org/10.3389/fmicb.2017.01919.