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Bacterial biota of shrimp intestine is significantly modified by the use of a probiotic mixture: a high throughput sequencing approach

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Abstract

The use of probiotics is a common practice of current shrimp aquaculture. Despite the immunophysiological responses that have been measured in shrimp exposed to probiotics, no information is currently available on the effect of this practice on the intestinal microbiota. The objective of this work was to evaluate the effect of a probiotic mixture on the intestinal microbiota of shrimp cultured under farm conditions. A culture-independent method based on high-throughput-sequencing (16S rRNA) was used to examine intestinal bacterial communities. A traditional system (without probiotics) was used as the reference. Targeted metagenomics analysis revealed that the probiotic mixture was based on bacteria in the phyla Proteobacteria and Firmicutes. A total of 23 species of bacteria were detected in the probiotic mixture; of these, 11 were detected in the intestine of shrimp reared in both systems, and 12 were novel for the system. Eight of the novel species were detected in shrimp cultured with the probiotic mixture; however, none of these novel species were related to marine or inclusively aquacultural environments, and only one (*Bacillus subtilis*) was recognized as probiotic for shrimp. The use of the probiotic mixture modified the bacterial profile of the shrimp intestine; however, most of the bacteria incorporated into the intestine were nonindigenous to the marine environment with no previous evidence of probiotic effects on any marine organism. The use of this probiotic mixture may represent a risk of causing environmental imbalances, particularly because farms using these types of probiotic mixtures discharge their effluents directly into the ocean without prior treatment.

Keywords: Aquaculture, Bacterial diversity, Intestinal microbiota, Metagenomics, Probiotics

Background

Pathogens have undermined shrimp aquaculture [1, 2], and for the past two decades, antibiotic use has been the most important and effective strategy to control and prevent bacterial infections [3, 4]. However, the use of antibiotics may have negative consequences for human and environmental health, inducing resistance to antibiotics and promoting transference of antibiotic resistance genes [5, 6].

A wide diversity of marine pathogens can develop plasmid-mediated resistance. For example, plasmids containing genes for antibiotic resistance have been detected in marine species associated with aquaculture [7, 8]. The presence and transfer of plasmids containing antibiotic resistance genes may cause a continuous decrease in antibiotic effectiveness, which is usually an expensive resource.

Therefore, strategies aimed at controlling disease and improving shrimp health are required. The use of probiotics is one of the most promising alternatives [7]. The

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consensus defines probiotics as living microorganisms that confer any health benefit on the host by diverse mechanisms, including improvement in intestinal balance, delivery of certain enzymes to the intestine for digestion purposes, production of chemicals that inhibit or decelerate the growth of possible pathogens, competition against pathogens for resources and/or enhancement of the immune response of the host [9, 10].

Therefore, probiotics can be introduced into the culture environment to control and compete with pathogenic bacteria and to promote the growth of the cultured organisms [11]. However, most of the research on the use of probiotics, including that of commercial products, has focused on the physiological and immunological responses of the host, in addition to host resistance to pathogen challenges and production performance [11–13].

Although these responses are important economic considerations; no extensive evidence regarding the effect of these products on the host microbiota is available, which is one of the primary targets of probiotics. Understanding the effects of probiotic use on intestinal microbiota is critically important to comprehend the effect of these strategies on shrimp aquaculture, considering the multiple guest–host functions in which intestinal microbiota participate (i.e., metabolic, trophic and protective functions) [2, 14]. Therefore, comprehension of the biology of a cultured organism may represent a guarantee of success. Furthermore, changes in microbiota induced by the use of probiotics may be beneficial to shrimp, but a risk also occurs that the changes will affect microbial communities with important functions for these crustaceans.

Probiotic products are used indiscriminately in very different environmental conditions [15]. However, this strategy may be inadequate given that some of the bacteria species contained in these products may not be adapted to the marine ecosystem and are most likely incapable of thriving in the conditions generated within shrimp/fish ponds. Finally, risks are also associated with the introduction of nonindigenous species into the marine ecosystem; for example, probiotics can contain plasmids with antibiotic resistance genes [15, 16]. Therefore, it is also important to identify the bacteria species that are introduced into open aquaculture systems.

The aim of this research was to study the effect of a probiotic mixture used in shrimp farms on the intestinal bacterial biota (16S rRNA) of shrimp.

Methods

Culture systems

Shrimp were cultured for two months in an industrial-scale shrimp aquaculture system located at the Quinta San Fabián farm (27°53′55.44″N, 110°38′6.52″W) at Empalme, Sonora, Mexico. The culture system was based on the traditional conditions for shrimp rearing (see Fig. 1) but with the daily addition of a previously prepared aerobic mixture of probiotics.

Shrimp (*Litopenaeus vannamei*) weighing 0.3 ± 0.04 g were stocked at 35 shrimp m^{-2} in 2 ha earthen ponds 1.5 m in depth. Organisms were fed twice a day at 4% of daily wet biomass·day^{-1} with commercial feed containing 35% crude protein (PURINA®). Water temperature oscillated between 25 and 32 °C, with dissolved oxygen (DO) at 4–6 mg L^{-1}, salinity at 38-40 PSU, and pH values of 7.9–8.6. Paddle aerators were used during the night, and a daily water exchange ratio of 10% was performed using water directly from the ocean. Twelve ponds were selected for the study: six using the probiotic mixture and six without addition of probiotics.

Probiotic production

Eight bioreactors (10,000 L each) were used at full capacity on the farm to promote the growth of probiotic bacteria. Bioreactors were designed to improve the proliferation of aerobic bacteria; the culture media was constantly mixed by axial flow, and air micro bubbles were injected at the bottom of the reactors.

Bioreactors were inoculated with a commercial probiotic product based on a Bacilli- and Gamma-proteobacteria mixture (Eco-AQUAPROTEC, Australia). The bacterial profile based on 16S rRNA (regions v3-v4) is presented below (Fig. 1). Briefly, the water used for the bioreactors was filtered by flow through one-micron sand filters and then was sterilized by eight UV lamps. Probiotic cultures were grown under constant light conditions (100 µmol photon/m^2/s, 500 nm) to enhance the proliferation of autotrophic bacteria. The bio-catalyzer design was based on the fermentation of organic material, including cow manure, vegetal substrate and other ingredients (not revealed by the company), aimed at producing oxygen and hypersaline-adapted enzymes. This phenomenon was achieved by maintaining a constant pressure (500 millibar) with hyperbaric columns on the side of the bioreactors.

A carbon:nitrogen:phosphorus ratio of 20:3:1 was maintained in each reactor by adding molasses as a carbon source (20 L), $NaNO_3$ (2 kg) and $(NH_4)_2SO_4$ (1 kg) as nitrogen sources, and KH_2PO_4 (0.5 kg) and K_2HPO_4 (0.5 kg) as phosphorus sources. The pH was up-regulated by the addition of NaOH (≤1 kg) 1 h before the inoculation (Na_2CO_3 was used during the bacterial culture). Physicochemical conditions were maintained at 30 °C, with dissolved oxygen at 4 mg L^{-1} and a pH of 7.5 (adjusted every 3 h). Once these conditions were met, the probiotic was inoculated at a density of ≈25,000 cells mL^{-1}. Bacterial cell counts reached

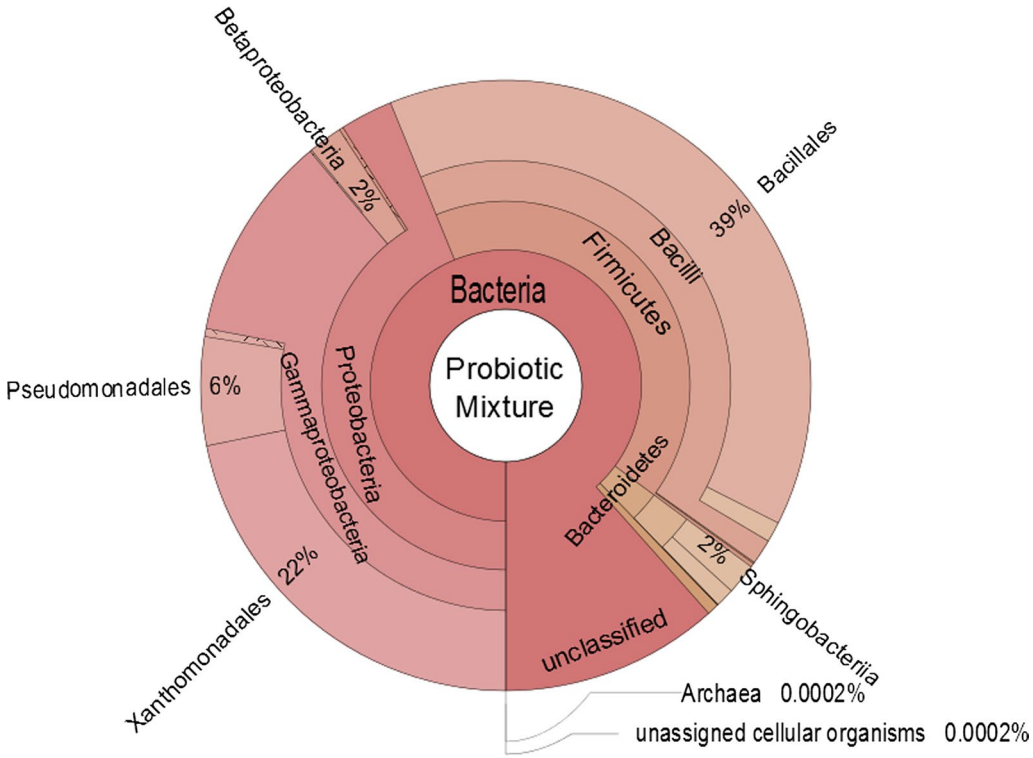

Fig. 1 Classification chart (KRONA) of the bacteria contained in the probiotic mixture used for shrimp aquaculture. Taxonomic classification is presented as Phylum, Class and Order (from inside to outside). Organisms representing less than 0.5% are not labeled in the chart. Organisms belonging to the bacteria domain but unable to be classified at a more refined taxonomic level are labeled as "unclassified" bacteria. Proportion of reads assigned to higher taxonomic levels is not specified in this figure (see Table 1)

the maximum concentration at 24 h, registering 1×10^{12} CFU mL^{-1} for the probiotic mixture. Once the maximum concentration was attained, the probiotic mixture was ready for use in the shrimp culture units.

A volume of 150 L of probiotic mixture was added directly to the water of each pond on the farm daily (except for the six reference ponds), and the reactors were prepared to amass bacteria again.

Bacterial diversity

The bacterial diversity of the probiotic mixture and shrimp intestines was monitored with a targeted loci approach on two sample dates using the 16S rRNA gene and considering the hypervariable regions V3 and V4. Alpha (α) and beta (β) diversity was calculated using a analytic hierarchy method described by Goepel [17] and using the Shannon-index and relative homogeneity values as parameters.

Shrimp intestines were obtained on the 30th and 60th days of culture. Six earthen ponds for each culture system were used for the study, and 10 shrimp intestines·$pond^{-1}$ were sampled on the above dates using 50-mL sterile falcon tubes (VWR, USA) and then stored at −80 °C. Intestine samples from the same ponds were pooled, and ponds were the replicates.

Nucleic acids isolation

DNA was isolated from the probiotic mixture and shrimp intestines. Intestines were dissected from the rest of the carcass and longitudinally cut; intestines were washed with sterile/nuclease-free water to remove fecal matter and transient-unattached microorganisms. Thereafter, the probiotic mixture and the intestine samples from each culture system were homogenized at 6 m s^{-1} in a FastPrep-24™ 5G homogenizer (MP Biomedicals, USA). The commercial Meta-G-Nome™ DNA isolation kit (Epicentre, USA) was used for the isolation of randomly sheared, high molecular weight metagenomic DNA (free of humic and fulvic acids) directly from unculturable or difficult-to-culture bacteria in shrimp intestines.

To isolate DNA from the probiotic mixture, 10 mL of the mixture was filtered through a 0.45-μm membrane (Millipore, USA); from this step forward, the instructions used with the above DNA isolation kit were strictly followed for all samples.

The quality of the DNA isolates was monitored with a 2200 Tapestation microfluidic electrophoresis instrument (Agilent, USA). Briefly, 1 µL of DNA isolate solution was mixed with 10 µL of gDNA sample buffer (Agilent, USA), and a gDNA Ladder (Agilent, USA) was used as the reference. From the mixture, 1 µL per sample was inserted into a microfluidic chip (gDNA ScreenTape, Agilent, USA) for the analysis of 200–>60,000-bp DNA fragments. Finally, the microfluidic chip containing the nucleotide samples was inserted into a 2200 Tapestation Electrophoresis Instrument (Agilent, USA). All the samples with DNA integrity numbers above 7 were considered for the following library preparation.

Library preparation

The library preparation was performed by strictly following the "16S-metagenomic sequencing library preparation guide" published by Illumina. The protocol consisted of the amplification of a 16S rRNA fraction containing the V3–V4 region [18] with sequencing adapters and dual index barcodes for the identification of samples (Nextera XT library preparation kit; Illumina, USA):

16S Amplicon PCR Forward Primer (Bakt_341F) + **Overhang adapter** = 5′-**TCGTCGGCAGCGTCAG ATGTGTATAAGAGACAG**CCTACGGGNGGCW GCAG-3′;
16S Amplicon PCR Reverse Primer (Bakt_805R) + **Overhang adapter** = 5′-**GTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAG**GACTACHVGGGTA TCTAATCC-3′.

A first amplification step was performed in 25-µL reactions using 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA) under the following thermal cycling conditions: one initial denaturation step at 95 °C for 3 min; 25 cycles at 95 °C for 10 s, at 55 °C for 30 s, and at 72 °C for 30 s; an extension at 68 °C for 5 min; and a hold step at 4 °C. Thereafter, the resulting amplicons (450–550 bp) were submitted to a cleanup procedure using magnetic AMPure XP beads (Beckman Coulter, USA) to purify the amplicons from free primers and primer dimer species, following the manufacturer's specifications. Once the amplicons were cleaned, they were attached with dual indices and sequencing adapters using the Nextera XT Index Kit (Illumina, USA) and 2× KAPA HiFi HotStart ReadyMix. The above thermal cycling conditions were performed for 8 cycles, and the above process using AMPure XP beads was repeated to again clean up the library. Finally, the resulting library was quantified and qualified by capillary electrophoresis (2200 Tapestation, Agilent, USA) using microfluidic chips (D1000 Screen Tape, Agilent, USA) with an analysis range of 35–1000 bp. Briefly, 2 µL of indexed library samples was mixed with 2 µL of High Sensitivity D1000 buffer. These samples were loaded into High Sensitivity D1000 screentapes and analyzed using the 2200 Tapestation instrument.

Sequencing

All the libraries were adjusted to a concentration of 4 nM using 10 mM Tris (pH 8.5) as diluent and pooled in the same proportion. Thereafter, libraries were denatured with 0.2 N NaOH. A standard PhiX Control Library (Illumina, USA) was also denatured and used as an internal control. Both the denatured library and PhiX Control were adjusted to a concentration of 8 pM and mixed (95% library + 5% PhiX Control). Finally, the mixture was heated to 96 °C for 2 min and immediately cooled on ice for 5 min.

The sample was then loaded into a MiSeq v3 Reagent Tray (Illumina, USA) and inserted into a MiSeq sequencing instrument (Illumina, USA), which contained a MiSeq v3 Flow Cell (Illumina, USA) with a capacity of 25 million reads. The results were obtained after 300 cycles.

Data analyses

All sequences were submitted to taxonomic classification. Ultrafast-metagenomic sequence classification using exact alignments (Kraken) was performed in the Illumina BaseSpace platform (basespace.illumina.com). A phylotype-based analysis was performed instead of using an OTU-based approach considering the superior classification performance reported in recent studies [19, 20]. Kraken maps each single sequence to the lowest common ancestor in the database and performs alignments of matching sequences, forming a subtree whose nodes have a specific weight equal to the number of sequences associated with the node's taxon; finally, the subtree is used for classification.

Data of relative abundance of each bacterial species were analyzed using a repeated-measures analysis of variance, considering culture system as the main factor in the hierarchical linear modeling, and a significance level of $p = 0.05$.

Results

A total of 2,482,431 reads were generated for the probiotic sample. Approximately 74% of the reads were classified to order, 40% to family, and ~35% to genus and species.

With respect to intestine samples, an average of 150,000 reads per sample was obtained from which ~60% were assigned to order, 45–55% to family, and 35–40% to genus and species.

Targeted metagenomics analysis of the probiotic mixture revealed that it was primarily composed of bacteria

in the phyla Proteobacteria (primarily order Gammaproteobacteria) and Firmicutes (primarily Bacilli) (Fig. 1) and a smaller proportion of bacteria from diverse phyla.

Alpha diversity values were 1.64 and 2.03 (Shannon-index) for shrimp reared in the traditional and the probiotic-based systems, respectively, with relative homogeneity values of 98.7 and 93.5%, respectively. Regarding β-diversity, the Shannon-index value was 0.36, and the relative homogeneity was 39.7%.

The taxonomic profile of the bacteria in the intestines revealed that some kinds of bacteria were common in shrimp and that the use of the probiotic mixture had a significant effect ($p < 0.05$) on the bacterial profile of the shrimp intestine (Figs. 2, 3, 4; Tables 1, 2). Striking differences between treatments were detected inclusively at the phyla level (Fig. 2); however, differences in the bacterial profile at the species level were also detected (Table 2; Figs. 3, 4).

Regarding phyla level, a high proportion of Proteobacteria was detected in the intestines of shrimp from both treatments. Higher proportions of Proteobacteria (particularly the class Alphaproteobacteria) and Bacteroidetes were detected in the intestines of shrimp reared in the probiotic-based system than in those of shrimp cultured without probiotics. Additionally, lower proportions of Planctomycetes, Actinobacteria and Firmicutes were detected in the intestines of shrimp from the probiotic-based system than in those of the traditional system (Fig. 3).

The most evident differences between treatments were detected in the fraction of reads assigned to a genus or species. For example, a total of 23 different species were detected in the probiotic mixture in different proportions (Table 1); of these, 11 were also detected in the intestines of shrimp reared in both systems, but 12 were completely novel for the systems. None of these novel species were detected in shrimp reared in the traditional system, but eight were detected in shrimp cultured with the probiotic mixture, which included *Methylomonas methanica*, *Pseudomonas stutzeri*, *Pseudoxanthomonas suwonensis* (all Proteobacteria), *Bacillus subtilis, Geobacillus thermoleovorans* (Firmicutes), *Sphingobacterium ingobacterium* sp. (Bacteriodetes), *Oceanithermus profundus* (Deinococcus-Thermus) and *Mycoplasma synoviae* (Tenericutes) (Table 1).

Apart from these species, a total of 19 species of bacteria in the phyla Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Planctomycetes, Chlamydiae and Cyanobacteria were detected in shrimp from both treatments, independent of the use of probiotics (Table 3).

No single species of bacterium was detected that was exclusive to shrimp reared in the traditional system; additionally, the bacterial profile was similar for these shrimp on the 30th and 60th days (Figs. 2, 4). However, modifications were detected in shrimp reared in the probiotic-based system on the 30th day relative to the same shrimp on the 60th day (Table 3; Fig. 3). For example, eight species of bacteria in the phyla Proteobacteria, Deinococcus-Thermus, Planctomycetes and Cyanobacteria and representing at least 1% of the reads increased or decreased by at least 50% from the 30th to the 60th day (Table 4). Additionally, bacteria species such as *Desulfurivibrio alkaliphilus*, *Pseudomonas fluorescens* and *Anaerococcus prevotti* were detected in these shrimp on the 30th day but were not detected on the 60th day. By contrast, *Micavibrio aeruginosavorus* and *Leadbetterella byssophila* were detected on only the 60th day (Table 4).

With respect to abundance, *T. whipplei, Rhodopirellula baltica, I. coccineus, M. adhaerens* and *P. acanthamoebae* represented approximately 95% of the reads assigned to the level of species detected in shrimp cultured within the traditional system, whereas *T. whipplei, R. baltica, Rhudobacter capsulatus, P. brasilensis, K. vulgare* and *P. stutzeri* represented 85% of the reads in shrimp from the probiotic-based system (Fig. 4).

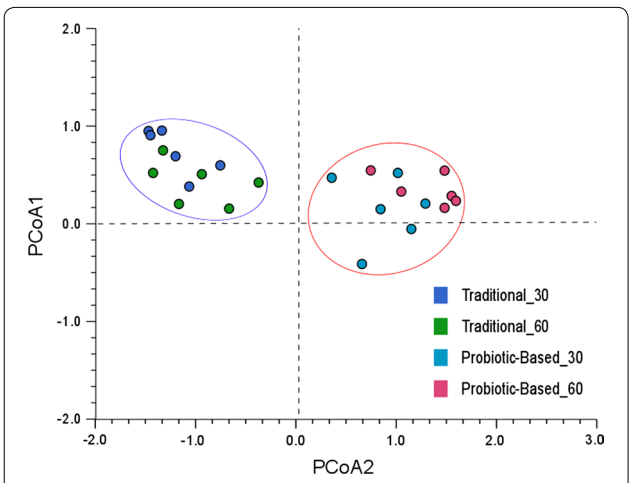

Fig. 2 Principal component analysis of the intestine microbiome at the phylum level detected in shrimp reared in the traditional and the probiotic-based systems on different culture dates (30 and 60 days). Ordination graph for the two axes of PCoA (PCoA1 < 10%, PCoA2 > 80%)

Discussion

Microbial identification in animal husbandry and agriculture is meaningful only when microbiota can be classified to the level of genus or species [21]. The classification of 35–40% of the reads to a genus or species was a successful result, considering that only a small fraction of all 16S gene sequences belonging to unculturable bacteria are registered in databases [22].

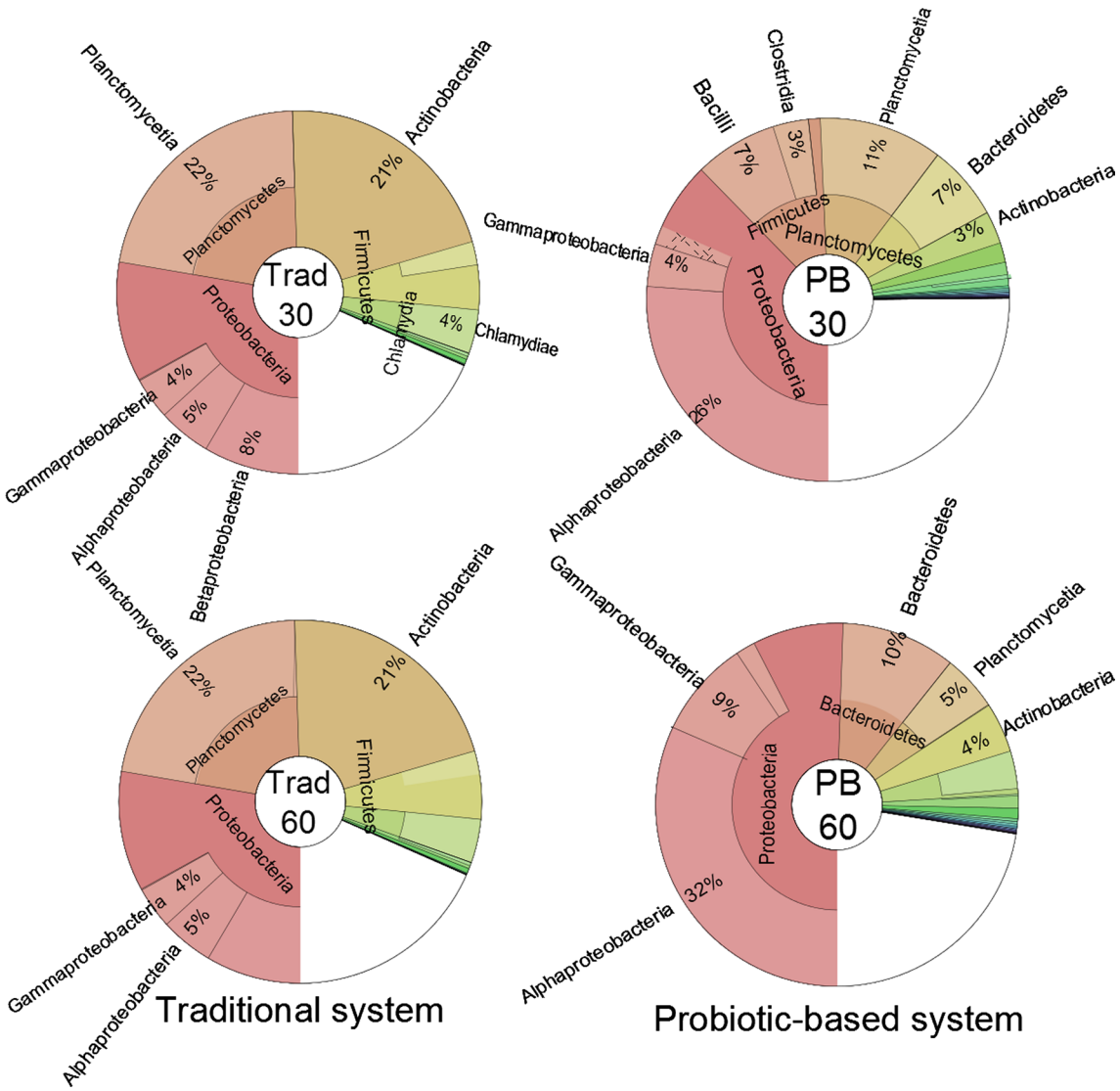

Fig. 3 Taxonomic profile of intestinal bacterial diversity of shrimp reared in the system using the probiotic mixture (PB) and shrimp reared without probiotics (traditional system). *Top charts* were obtained from shrimp on the 30th day of culture, whereas *bottom charts* were from the 60th day. The *white* unlabeled fraction represents sequence reads that belonged to the bacteria domain but were unable to be classified at a more refined taxonomic level. Graphics consider the mean values of all repetitions

Alpha diversity analysis revealed that the bacterial profile was uniform within samples of both treatments, whereas β-diversity and relative homogeneity demonstrated that the use of probiotics had an effect on the intestinal microbiota. For example, probiotic use favored the growth of Proteobacteria and inhibited that of Planctomycetes.

Proteobacteria, Planctomycetes, Actinobacteria, Firmicutes and Chlamydiae colonized the intestines of shrimp reared without probiotics, but the probiotics favored the growth of Proteobacteria and inhibited that of Planctomycetes. Most of these phyla, except for Planctomycetes, have been previously reported as natural microbiota of the intestines of penaeid shrimp [2, 14].

Although the colonization of novel bacteria species in the intestines of shrimp from the probiotic-based system could represent a good indicator of probiotic functioning, these new bacteria and the associated modifications of the microbiota of the intestines must be analyzed. Several bacterial species contained in the probiotic that also colonized the intestinal tract of shrimp are currently not associated with the marine environment and have not been recognized as probiotics. For example, *M. methanica* is considered a methanotrophic symbiont that thrives

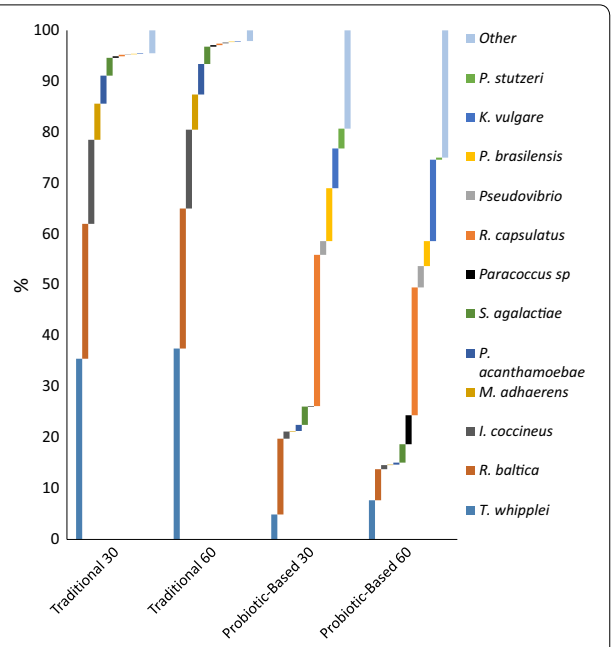

Fig. 4 Proportion of reads (represented at least at 0.5%) classified exclusively to genus or species levels detected in the intestines of shrimp reared with (Probiotic-Based) and without probiotics (Traditional) on the 30th and 60th days of culture. Reads classified to more superficial taxonomic levels are not included. Graphics consider the mean values of all repetitions

in both freshwater and marine ecosystems but particularly in methane-producing sludge [23]. Although toxic compounds such as hydrogen sulfide and methane are produced in the bottom of shrimp ponds, this risk can be eliminated by adequate management practices. Therefore, there is no evidence that this type of bacterium could promote any probiotic effect in shrimp intestines. *P. suwonensis* is an organophosphate- and cellulose-degrading bacteria commonly detected in woody environments [24]. *Sphingobacterium* sp. is a gram-negative, strictly aerobic, chemoorganotrophic bacterium that has been isolated from terrestrial environments and aerobic sludge and is capable of degrading acetanilide herbicide [25]; some strains have been detected in fish aquaculture systems, but their role remains unclear. *Mycoplasma synoviae* is a gram-negative bacterium pathogenic for several poultry species, and there are no reports associating this species with any marine animal or environment. *Oceanithermus profundis* is a gram-negative, thermophilic, organotrophic, chemolithotrophic bacterium isolated from deep-sea hydrothermal vents [26]. Some of these bacteria are commonly used for their biodegradation properties; however, there is no evidence that they have any probiotic effects in shrimp or other marine animal.

Among the bacteria that colonized shrimp intestine, *P. stutzeri* reportedly participates in denitrification processes, reducing nitrate to dinitrogen gas [27]. This Proteobacteria demonstrates inhibitory effects against *Vibrio parahemolitycus* (ATCC 17802), *V. alginolyticus* (ATCC 17749) and *V. alginolyticus* (S1) in aquatic organisms [28]. *B. subtilis* is perhaps the only one of these species that is recognized as a probiotic for penaeids because of inhibitory effects against some pathogens [29]. *G. thermoleovorans* is an extreme thermophile with antibacterial activity against human pathogens that possesses enzymes such as phosphatase alkaline, esterase lipase, amylase, lipase, lecitenase and caseinase [9]; however, it is not known whether this bacterium has any antibacterial effects on marine pathogens.

It is possible that some of these bacteria (most of them not from a marine environment) are capable of colonizing shrimp intestines because of their physiological plasticity. Some of these bacteria have been isolated from extreme environments. Despite the successful colonization of some of these bacteria in the intestine, bacteria thriving in the immediate environment of marine species may have a much greater influence on the health and pathogen resistance of shrimp than that of allochthonous bacteria whose colonization is achieved by the constant addition of the probiotic mixture. These results revealed a possible alarming scenario because most shrimp farms include open systems, and farmers using this type of product or performing similar practices may be introducing nonindigenous bacterial species into the marine environment.

The effect of the probiotic mixture on the intestinal microbiota must be analyzed based on the idea of Mahdhi et al. [9], who argued that *"probiotics are live microbial feed supplements, improving the intestinal balance of the host and producing metabolites which inhibit the colonization or growth of other microorganisms or by competing with them for resources such as nutrients or space."* In this regard, a modification of the microbiota (presence and abundance) was detected when shrimp were exposed to the probiotic mixture; however, the abundance of some marine and native bacteria decreased in shrimp exposed to the probiotic mixture. For example, the proportion of *R. baltica,* which is a marine aerobic heterotrophic bacterium usually detected in aquaculture farms and marine environments that plays a role in the nitrogen cycle and biodegradation of organic material, decreased [30]. By contrast, *R. capsulatus,* which possesses a wide range of metabolic capabilities and some antibacterial activity against gram-negative bacteria [31], increased. Additionally, *Pseudovibrio* sp., which has some antibacterial activity against marine sponge pathogens also increased.

It is difficult to conclude whether these changes in bacterial proportions corresponded to a positive or to a negative effect of the probiotic mixture. Earlier experiments

Table 1 Bacteria species detected in the probiotic mixture and their detection in shrimp reared in the probiotic-based system (using the mixture) and in the traditional system

Phylum	Species detected in the probiotic mixture	Proportion (%)	Traditional system	Probiotic-based system
Proteobacteria	*Acinetobacter baumannii*	3.2	−	−
	Methylomonas methanica	0.8	−	+
	Pseudomonas stutzeri	1.6	−	+
	Pseudoxanthomonas suwonensis	1.6	−	+/−
Firmicutes	*Bacillus subtilis*	0.8	−	+/−
	Geobacillus thermoleovorans	41.6	−	+
	Macrococcus caseolyticus	1.6	−	−
	Sphingobacterium ingobacterium sp.	3.2	−	−/+
	Weeksella virosa	12.8	−	−
Bacteriodetes	*Pedobacter saltans*	0.8	−	−
Deinococcus-Thermus	*Oceanithermus profundus*	1.6	−	+
Tenericutes	*Mycoplasma synoviae*	1.6	−	+
Proteobacteria	*Colwellia psychrerythraea*	1.6	+	+
	Ketogulonicigenium vulgare	0.8	+	+
	Salmonella enterica	1.6	+	+
	Xanthomonas albilineans	1.6	+	+
Firmicutes	*Bacillus cereus*	4.2	+	+
	Bacillus thuringiensis	3.8	+	+
	Bacillus weihenstephanensis	0.8	+	+/−
	Streptococcus agalactiae	11.2	+	+
Actinobacteria	*Tropheryma whipplei*	0.8	+	+
	Nocardioides ardioides sp.	0.8	+	+
Spirochaetes	*Borrelia relia* sp.	1.6	+	+/−

Detected (+), Detected at the 30th or 60th day of culture only (±), Not detected (−). The proportion of reads detected for each species is also included

Table 2 Proportion of reads (v3–v4, 16S rRNA) assigned to species level in samples of shrimp reared in the traditional system (no probiotics) and in the probiotic-based system

	Traditional		Probiotic-based		Culture system		Time	
	30 days	60 days	30 days	60 days	p value	F-ratio	p value	F-ratio
T. whipplei	35.5 ± 1.9	37.5 ± 1.1	4.9 ± 0.6	7.7 ± 0.5	0	746	0.06	4.78
R. baltica	26.5 ± 1.2	27.5 ± 0.6	15.0 ± 1.0	6.1 ± 0.2	0	786.2	0.02	2.99
I. coccineus	16.5 ± 0.6	15.5 ± 0.8	1.4 ± 0.3	0.8 ± 0.1	0	795.5	0.19	2.01
M. adhaerens	7.1 ± 0.1	6.9 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	0	3559.1	0.67	0.19
P. acanthamoebae	5.5 ± 0.7	6.9 ± 0.3	1.2 ± 0.1	1.4 ± 0.6	0	97.89	0.1	3.4
S. agalactiae	3.5 ± 0.3	3.4 ± 0.2	3.6 ± 0.3	3.6 ± 0.1	0.62	0.26	0.8	0.07
Paracoccus sp.	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	5.7 ± 0.4	0	185.8	0	216.1
R. capsulatus	0.3 ± 0.1	0.3 ± 0.1	29.7 ± 2.1	25.1 ± 0.7	0	638.1	0.07	4.45
Pseudovibrio	0.1 ± 0.1	0.3 ± 0.1	2.7 ± 0.2	4.2 ± 0.3	0	400.2	0	27.4
P. brasilensis	0.1 ± 0.1	0.1 ± 0.0	10.4 ± 1.3	5 ± 1.1	0	85.2	0	10.7
K. vulgare	0.1 ± 0.1	0.1 ± 0.1	7.9 ± 1.2	16 ± 1.5	0	161.3	0	18.6
S. stutzeri	0 ± 0.00	0 ± 0.00	3.9 ± 0.6	0.7 ± 0.2	0	57.2	0	25.6
Other	4.5 ± 0.7	1.2 ± 0.5	19.3 ± 1.3	23.6 ± 1.5	0	310.1	0.7	0.16

Table describes only the most abundant species detected in both treatments. Deviation indicates standard error

Table 3 Bacteria species detected exclusively in shrimp reared in the traditional and probiotic-based systems on the 30th and 60th days

Phylum	Species
Proteobacteria	*Alteromonas macleodii*
	Marinobacter adhaerens
	Nitrosospira multiformis
	Paracoccus sp.
	Pseudovibrio sp.
	Rhodobacter capsulatus
	Rhodospirillum photometricum
	Vibrio sp.
Firmicutes	*Acetohalobium arabaticum*
	Clostridium botulinum
	Tetragenococcus halophilus
Bacteriodetes	*Cytophaga hutchinsonii*
	Riemerella anatipestifer
Actinobacteria	*Ilumatobacter coccineus*
Planctomycetes	*Planctomyces brasiliensis*
	Rhodopirellula baltica
Chlamydiae	*Parachlamydia acanthamoebae*
	Simkania negevensis
Cyanobacteria	*Synechococcus* sp.

Table 4 Changes in the intestinal bacterial profile of shrimp reared in the probiotic-based system on the 30th and 60th days

Phylum	Species
Increase	
Proteobacteria	*Alteromonas macleodii*
	Ketogulonicigenium vulgare
	Paracoccus sp.
Decrease	
Proteobacteria	*Pseudomonas stutzeri*
Deinococcus-Thermus	*Oceanithermus profundus*
Planctomycetes	*Planctomyces brasiliensis*
	Rhodopirellula baltica
Cyanobacteria	*Parachlamydia acanthamoebae*
Detected on the 30th day only	
Proteobacteria	*Desulfurivibrio alkaliphilus*
	Pseudomonas fluorescens
Bacteriodetes	*Anaerococcus prevotti*
Detected on the 60th day only	
Proteobacteria	*Micavibrio aeruginosavorus*
Bacteriodetes	*Leadbetterella byssophila*

The table shows species of bacteria whose proportion was at least 1% and that exhibited a change (increase or decrease) of at least 50% of their value from the 30th to 60th day. Other data shown includes the detection of bacteria on the 30th day but not detected on the 60th day and vice versa

in our laboratory revealed that this probiotic mixture enhances some of the immune responses of shrimp, but it was not possible to elucidate which of the tens of different bacteria contained in the mixture caused the effect [25]. Moreover, enhancing immune responses with non-indigenous species is not a guarantee of health and could require an unnecessary expenditure of energy.

For shrimp reared with probiotics, the differences between the 30th and 60th day suggested that a lower dose of probiotics should be used. The modification of microbiota may favor the proliferation of other species, as was observed in this experiment; however, the constant addition of bacteria (mostly nonindigenous) to shrimp also entails a constant addition to the marine environment.

None of the bacteria identified (except for the genus *Bacillus*) is recognized in the literature as a probiotic for crustaceans. As stated above, probiotic mixtures should be based on microorganisms isolated from similar and preferably immediate environments to the host.

A common strategy to obtain this type of bacteria is to perform in vitro antagonism tests, exposing pathogens to alleged probiotics. Although this in vitro strategy may not provide 100% confidence because some probiotics may behave differently in vivo and in aquaculture systems, this strategy could be a first approach to study the identified candidates. Other approaches may include the exposure of hosts reared with particular probiotics to pathogen challenges.

The bacterial diversity observed in shrimp, both cultured with and without probiotics, revealed that species in the phyla Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Planctomycetes, Chlamydiae and Cyanobacteria composed the regular microbiota of shrimp; some of these may constitute first case reports for the species.

Finally, one notable concern was that some human bacterial pathogens were identified, including *T. whipplei*, *P. acanthamoebae* and *S. enterica,* which are usually detected in human feces, wastewater, and contaminated food. Although whether the bacteria detected corresponded to pathogenic strains could not be determined, this finding constitutes a first case report of this type of bacteria (in addition to others in this study) in shrimp aquaculture systems. One possible explanation for these bacteria is the practice of open farms performing water exchanges by taking water directly from the ocean without previous treatment.

Abbreviations

16S rRNA: ribosomal 16S RNA gene; CFU: colony forming units; DO: dissolved oxygen; gDNA: genomic DNA.

Authors' contributions
MPC, MMP and FVA conceived the study; MPC, EVC and TGG performed the experiment, sampling and all the analyses and handling of data. MPC, MMP, FVA, TGG and LRMC contributed to data interpretation. MMP and LRMC wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
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