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Research Article

Probiotic modulation of the gut bacterial community of juvenile *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* CAIM 170

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ABSTRACT. The protective effects of two probiotic mixtures was studied using the fingerprints of the bacterial community of *Litopenaeus vannamei* juveniles exposed to probiotics and challenged with *Vibrio parahaemolyticus* CAIM 170. Fingerprints were constructed using 16S rRNA gene and the PCR-SSCP (Single strand conformation polymorphism) technique, and the probiotics used were an experimental *Bacillus* mixture (*Bacillus tequilensis* YC5-2 + *B. endophyticus* C2-2 and YC3-B) and the commercial probiotic Alibio. The DNA for PCR-SSCP analyses was extracted directly from the guts of shrimps treated for 20 days with the probiotics and injected with 2.5×10^5 CFU g^{-1} of *V. parahaemolyticus* one week after suspension of the probiotic treatment. Untreated shrimps served as positive (injected with *V. parahaemolyticus*) and negative (not injected) controls Analysis of the bacterial community carried out after inoculation and 12 and 48 h later confirmed that *V. parahaemolyticus* was present in shrimps of the positive control , but not in the negative control or treated with the probiotic mixtures. A significant difference in the diversity of the bacterial community was observed between times after infection. The band patterns in 0-12 h were clustered into a different group from that determined after 48 h, and suggested that during bacterial infection the guts of whiteleg shrimp were dominated by gamma proteobacteria represented by *Vibrio* sp. and *Photobacterium* sp. Our results indicate that the experimental and the commercial mixtures are suitable to modulate the bacterial community of *L. vannamei* and could be used as a probiotic to control vibriosis in juvenile shrimp.

Keywords: *Litopenaeus vannamei*, *Bacillus* mix, *Vibrio parahaemolyticus*, bacterial community, aquaculture.

Modulación por probióticos de la comunidad bacteriana intestinal de juveniles de *Litopenaeus vannamei* infectados con *Vibrio parahaemolyticus* CAIM 170

RESUMEN. Se estudiaron los perfiles de bandeo de la comunidad bacteriana de juveniles de *Litopenaeus vannamei* tratados con dos probióticos y expuesto a la bacteria patógena *Vibrio parahaemolyticus* CAIM 170. Los perfiles de bandeo se construyeron usando el gen 16S rRNA y la técnica PCR-SSCP (Polimorfismo conformacional de cadena sencilla) y los probióticos fueron una mezcla experimental de *Bacillus* (*Bacillus tequilensis* YC5-2 y *B. endophyticus* C2-2 y C3-B) y el probiótico comercial Alibio. El ADN para el análisis PCR-SSCP se obtuvo de los intestinos de camarones tratados durante 20 días con los probióticos, inyectados con $2{,}5\times10^5$ UFC g^{-1} de *V. parahaemolyticus* una semana después de la suspensión del tratamiento con probióticos. Camarones no tratados con probióticos sirvieron como control positivo (inyectados con *V. parahaemolyticus*) y negativo (no inyectados). El análisis de la comunidad bacteriana durante el reto confirmó la presencia del patógeno inyectado en el control positivo y su ausencia en el negativo y en los organismos tratados con probióticos. Durante las 48 h del período experimental se observó una diferencia significativa en la diversidad de la comunidad bacteriana.

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Los patrones de bandas se agruparon en un grupo a las 0-12 h y en uno diferente después de 48 h y sugirieron que los intestinos de camarón blanco fueron dominados por gamma proteobacteria representados por *Vibrio* sp. y *Photobacterium* sp. durante la infección bacteriana. Estos resultados indican que las dos mezclas pueden modular la comunidad bacteriana y pueden ser usadas como probióticos para controlar la vibriosis en camarones juveniles.

Palabras clave: *Litopenaeus vannamei*, mezcla de *Bacillus*, *Vibrio parahaemolyticus*, comunidad bacteriana, acuicultura.

INTRODUCTION

The bacterial genus *Vibrio* is common and widely distributed in the natural marine environment and in the microbiota of farmed shrimp ponds (Gopal *et al.*, 2005), where some of its species may become opportunistic pathogens and sources of major diseases when the natural defense mechanisms of cultured shrimp are suppressed (Lightner, 2005).

Under the common name of vibriosis, these diseases may cause considerable economic losses, and are considered among the most serious limiting factors for the success of marine aquaculture (Lightner, 2005; Chatterjee & Haldar, 2012). Among the etiological agents, *Vibrio harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. campbelli*, *V. alginolyticus* and *V. penaeicida* have been associated with cultured shrimp diseases (Ishimaru *et al.*, 1995; Sahul-Hameed *et al.*, 1996; Jayasree *et al.*, 2006), and among these *V. harveyi* and *V. alginolyticus* are thought to be the most common causes of disease during larval and postlarval development (Manefield *et al.*, 2000; Abraham & Palaniappan, 2004).

The addition of probiotic bacteria to culture systems has gained attention as a precautionary measure against pathogens. This addition aims to reduce or eliminate selected pathogenic species and to improve growth and survival of the cultured species through the modulation of the microbial communities of the culture environment (Balcázar *et al.*, 2006; Martínez-Cruz *et al.*, 2012), because bacteria may affect growth and survival of aquatic organisms and are a major element in their well being, since they play distinct roles in the host organism, which are associated with nutrition, immune responses and disease resistance (Austin, 2006; Chaiyapechara *et al.*, 2011; Tuyub-Tzuc *et al.*, 2014).

Culture-independent techniques for population fingerprinting, such as denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP), are effective tools for a more complete and rapid assessment of microbial diversity, especially of complex ecosystems such as intestinal microbiota (Muyzer & Smalla, 1998; Dohrmann & Tebbe, 2004; Hassan, 2012).

Previous experiments showed that a *Bacillus* mixture which improved survival and development of *Litopenaeus vannamei* larvae caused also an increase in diversity and evenness of the bacterial community of the larval gut, thus increasing resistance to *V. parahaemolyticus* infection (Luis-Villaseñor *et al.*, 2011, 2013). However, there is no information on the effect of this or other probiotics on the structure of the bacterial community of the intestinal tract of juvenile or adult shrimp challenged with pathogenic bacteria. This study aimed to evaluate the changes induced by the same *Bacillus* mixture on the gut bacterial community of juvenile *Litopenaeus vannamei* (Pacific whiteleg shrimp) infected with *V. parahaemolyticus*.

MATERIALS AND METHODS

Probiotic strains

Cultures of the bacteria *Bacillus tequilensis* YC5-2, *B. endophyticus* C2-2 and *B. endophyticus* YC3-B were grown at 37°C for 24 h in 200 mL Erlenmeyer flasks with 100-mL of TSB medium, and concentrated by centrifugation at 5000×*g* for 10 min. Each pellet was suspended in a sterile saline solution containing 3% (w/v) NaCl (S-7653, Sigma, St. Louis, MO). The absorbance was adjusted to an optical density of 1 at 600 nm (approximately 1×10^9 CFU mL^{-1}), and the resulting suspensions were added to the shrimp rearing system at a final concentration of 1×10^5 CFU mL^{-1}.

Pathogenic bacterium

Strain *Vibrio parahaemolyticus* CAIM 170, obtained from the Colección de Microorganismos de Importancia Acuicola (CIAD, Mazatlan, Mexico, www.ciad.mx/caim), grown in trypticase soy broth (TS#236950, Difco, Franklin Lakes, NJ) with 3% (w/v) NaCl, was centrifuged at 5000×*g* for 10 min; the pellet was suspended in 3% (w/v) sterile saline solution. The bacterial suspension was diluted with filtered sterile seawater to an optical density of 1.0 (approximate concentration: 1×10^9 CFU mL^{-1}), and a 1:10 dilution of this suspension was used for the challenge experiment.

Probiotic treatment and infection

Juvenile shrimps (mean live weight 8 ± 1 g) were obtained from a commercial hatchery and acclimated

for five days to laboratory conditions, which did not change throughout the experiment (5-µm filtered seawater, 29°C and salinity 36) in a common tank. After acclimation, 16 groups of 21 shrimps were placed in 80-L aquaria. Five aquaria (treatment A) were added daily 1×10^5 CFU mL^{-1} of the *Bacillus* mixture. A second group of five aquaria (treatment B) received the dose used by local shrimp farmers (1 mL L^{-1}, with 1×10^6 CFU mL^{-1}) of a commercial probiotic mixture (Alibio2135 + AlibioAC + Alibio Bionutre) activated as recommended by the manufacturer (AliBio S.A. de C.V., Mexico City). The remaining six aquaria served as triplicate positive and negative (unchallenged) controls (treatments C and D, respectively).

Addition of probiotics was suspended after 20 days, and seven days later all shrimps of treatments A, B and C were injected into the fifth abdominal section with 20 µL of *Vibrio* suspension (= 1×10^8 CFU mL^{-1}), giving $2x10^6$ CFU/shrimp. Shrimps of treatment D were injected with a sterile saline solution.

Throughout the experiment all treatments were fed *ad libitum* a 35% protein commercial diet. Continuous aeration was maintained in all aquaria, which were maintained with 50% daily water exchanges.

Sample collection and DNA extraction

One shrimp was randomly selected from each container immediately after *Vibrio* injection (time 0) (5 shrimps for each probiotic treatment and 3 shrimps for positive and negative controls), and sampling was repeated after 12 and 48 h, in the first case because this time coincided with the first case of mortality, while the last was observed 12 h later. Consequently, samples of live shrimp were taken at times 12 h (onset of mortality) and 48 h, giving the surviving shrimp 24 to recover after the last observed death. Immediately after sampling, the body surface of each shrimp was washed with sterile seawater, disinfected with 70% ethanol, dissected with sterile instruments and the entire intestinal tract was removed, excised with sterile forceps and scissors, and preserved at -80°C in individual screw-capped tubes with 1 mL absolute ethanol.

At the end of the experiment, the chromosomal DNA was extracted to assay for the diversity of the intestinal communities, using Wizard genomic DNA purification kits (Promega, Madison, WI) according to the manufacturer's instructions.

Amplification of 16S rRNA

The universal bacterial primers Com1 and Com2ph were used to amplify a 407 bp fragment corresponding to positions 519 to 926 (*E. coli* positions; including variable regions 4 and 5 of the 16S gene). The Com1 sequence was 5′-CAGCAGCCGCGGTAATAC and Com2ph was 3′-CCGTCAATTCCTTTGAGTTT (Schwieger & Tebbe, 1998). Each PCR was performed in a total volume of 50 µL in 0.2 mL micro tubes. The reaction mixtures were contained in 1×PCR buffer with 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP, and 2.5 U Taq polymerase (GoTaq, Promega). The total amount of genomic DNA added to the PCR mixtures was 250 ng. Thermocycling (Peltier Thermal Cycler, Bio-Rad Laboratories, Hercules, CA) started with an initial denaturation for 3 min at 94°C, followed by 30 cycles of 60 s at 94°C, one cycle for of 60 s at 53°C and one of 90 s at 72°C, ending with a final extension for 5 min at 72°C. The presence of specific PCR products was confirmed on 1% (w/v) agarose gel.

Single-strand conformation polymorphism

The single-strand removal method (Schwieger & Tebbe, 1998) was used for profiling bacterial communities. All PCR products of each replicate were purified (PCR purification kit, Qiagen, Hilden, Germany) and diluted in Tris-HCl buffer to a final volume of 20 µL. Samples were digested for 45 min at 37°C with 1 µL (5 U) of lambda-exonuclease solution (New England Biolabs, Ipswich, MA), with 3 µL exonuclease buffer and 6 µL milli-Q H_2O, for a total volume of 30 µL. Digestion was stopped with the first step of purification with spin columns (MiniElute Kit, Qiagen), and diluted in 10 µL Tris-HCl buffer.

A 9 µL denaturing loading buffer containing 95% formamide (v/v), 10 mM NaOH (w/v), 0.25% bromophenol blue (w/v), and 0.25% xylene cyanole (w/v) was added before electrophoretic analysis. Samples were incubated at 95°C for 2 min and immediately cooled on ice. After 3 min, samples were loaded onto polyacrylamide gels of 0.625% MDE (Cambrex, East Rutherford, NJ), and electrophoresis at 260 V at 20°C was carried out for 18 h (DCode Universal Mutation System, Bio-Rad Laboratories, Hercules, CA). After electrophoresis was completed, the gel was stained with $AgNO_3$ (Benbouza *et al.*, 2006) and scanned using Power Look III (Umax Systems, Willich, Germany).

Analysis of SSCP profiles

Gel analysis software (Gel Compar II, Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate similarities between profiles of bacteria obtained from the different treatments and times of inoculation, after image normalization with bacteria markers (*B. licheniformis*, *Rhizobium trifolii*, *Flavobacterium johnsoniae*, and *R. radiobacter*). The calculation of the similarity matrix was based on Pearson's correlation

coefficients. The clustering method was the unweighted pair group method with arithmetic averages (UPGMA).

Elution of bands and DNA sequencing

Bands of interest were cut from the silver-stained polyacrylamide SSCP gel with a sterile scalpel. The single-stranded DNA was eluted from the gel by the crush and soak procedure (Sambrook & Russell, 2001), resuspended in 12 µL Tris buffer (10 mMTris-HCl, pH 8.0), and amplified via PCR using primers Com1 and Com2ph under the conditions previously described. The PCR-amplified products were sequenced by a commercial firm (Genewiz, South Plainfield, NJ).

The sequences were compared with sequences in the GenBank database. The BLAST search of the National Center for Biotechnology Information and the EzTaxon server database (www.eztaxon.org; Chun *et al.*, 2007) were used to determine the closest relationships of the 16s rRNA sequences.

Statistical analysis

To determine the similarity between treatments, the data of the metrics obtained from each sample were exported as a binary matrix (PAST software, palaeo-electronica.org). A PCA was performed from the correlation matrices generated from a binary matrix, which was expressed as a value of Pearson's similarity coefficient (Fromin *et al.*, 2002). A PCA analysis was conducted with software Statistica 6.0 (StatSoft, Tulsa, OK).

RESULTS

Modulation of intestinal microbiota

The dendograms showed a clear modulation of the intestinal microbiota from 12 h post-infection (onset of death) to 48 h (organisms recovered), divided into two clusters. One cluster (48 h) had a similarity value of 40.81% with respect to the second cluster, including times 0 and 12 h, with a percentage of similarity between 49.64%. Samples Start 1 and Start 2 (samples taken before probiotic treatment) were within the same cluster as time 48 h with 54% similarity, indicating a recovery of the initial microbiota similar to the bioassay (Fig. 1).

Bacterial community of juvenile shrimp infected by *Vibrio parahaemolyticus*

The results from the SSCP fingerprint showed that the taxonomic group Flavobacteria was dominant: α proteobacteria (mainly *Rugeria lacuscaerulensis*), γ-proteobacteria, fusobacteria and Cytophagaceae, represented by *Wandonia haliotis* Haldis.

V. parahaemolyticus and *Vibrio* sp. were present only in the positive control, *Cytophaga fermentans* was present only in organisms treated with Alibio while *Photobacterium damselae* subs. *piscicida* was present in all treatments but not in the positive control. The individual bands present in the *Bacillus* mix were identified as Candidatus bacilloplasma mollicute and *Nautella italica* (Table 1). Unidentified bands (Uncultured bacteria) were also present in the treatment of the *Bacillus* mix.

Maribius salinus and *Donghicola eburneus*(α-proteobacteria) were detected only in the *Bacillus* mix and the positive control groups (Table 1). Bacteria species unique to the negative control were *Thioprofundum lithotrophica*, *Sebaldella termitidis*, *Elizabethkingia anophelis*, *Oceanicola* sp., and *Thioclava pacifica*. *Thalassobium* sp. was detected in both control groups.

PCA Analysis

Principal Component Analysis (PCA) showed that two of the components explained 91% of the total variance in the data (CP1 and CP2: 64.8 and 26.2%, respectively) (Fig. 2), and that their factor loadings were considered significant at values greater than 0.70. No significant differences were observed between the banding profiles at time 0 h and 12 h, but their trends are separated clearly from that determined after 48 h. These results coincide with the indications of the similarity dendrogram.

DISCUSSION

Manipulation of microbiota with probiotics may be a convenient practice to control or inhibit pathogenic bacteria in aquaculture, as well as to improve growth performance and digestive enzymes activities, and enhance immune responses against pathogens or physical stress (Balcázar *et al.*, 2006; Pérez *et al.*, 2010; Zokaeifar *et al.*, 2012).

The *Bacillus* mix used in this work showed several effects which may be useful for *L. vannamei* culture: after a *V. parahaemolyticus* challenge which caused >90% mortality in the control group, juvenile shrimps treated with this mix had significant higher survival, different total hemocyte concentrations and a higher diversity and evenness of their bacterial gut community than those treated with the commercial product Alibio, and demonstrated efficient probiotic protection (Luis-Villaseñor *et al.*, 2013). However, the underlying mechanisms for this protection remained unclear.

In this work we showed how the effect of *V. parahaemolyticus* CAIM 170 on the bacterial community of juvenile shrimp may be at least partially avoided in shrimps treated with the *Bacillus* mix even

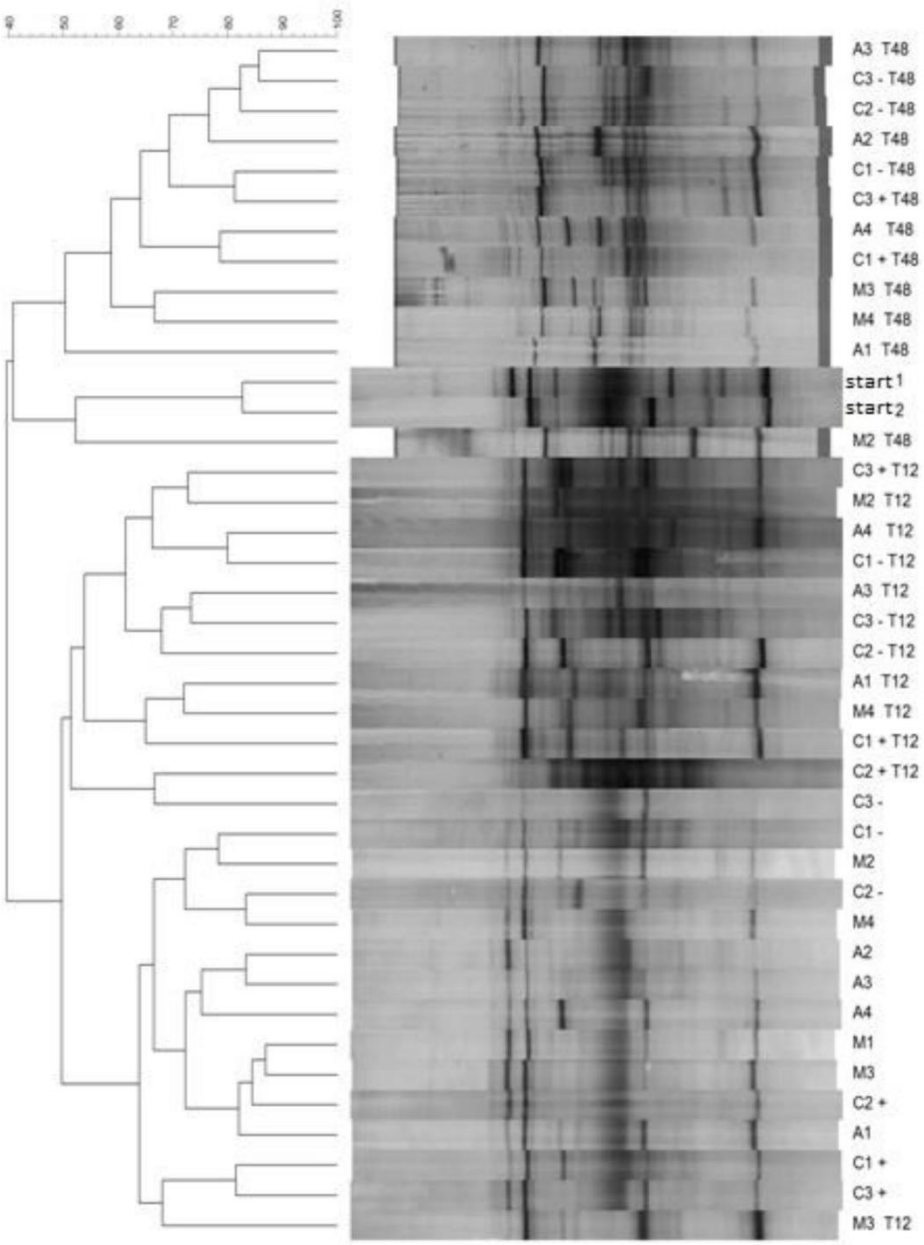

Figure 1. Acrylamide gel-generated via SSCP dendrogram illustrating the relationship (percent similarity) between bacterial communities in gut of shrimp at time 0 h, 12 h and 48 h: M1-M5 (*Bacillus* mix), A1-A5 (commercial probiotic), C1(-)-C3(-): (without probiotics and injected with saline solution) C1(+)-C3(+): (without probiotics and injected with pathogenic bacteria). Start 1 and 2: initial profiles, before probiotic treatment. The 40-100 scale of the dendrogram shows percent of similarity of the clusters. The dendrogram was calculated with UPGMA and the Dice coefficient.

after one week after suspension of the treatment. Although the SSCP distribution profiles displayed little variation of the number of bands (OTU) at each sampling period, the greatest variation observed was their increase 12 h after infection in all treatments with *Vibrio*.

The PCA of 0 h and 12 h indicated no evidence of clustering of individual probiotics groups and no statistically significant deviation from the baseline SSCP profile. However, the PCA conducted on the 48 h gut samples showed the probiotic groups clustered separately from those at the beginning and those at 12 h post-infection. This indicated that the gut microbial population ecology of the animals at 48 h was significantly different from that at 0 h and 12 h after infection, and the separation more evident was that between probiotic-treated and *V. parahaemolyticus*-infected shrimps.

Table 1. Closest relative, as determined by Blast search, with similarity (SIM, in %) to the major OTUs from the 16S rRNA V4 and V5 SSCP gels.

OTU	SIM (%)	Closest relative	Phylogenetic group
		Bacillus mix	
		h 0	
M1b	96	*Nautella italica*	α-proteobacteria
M1d	90	Candidatus bacilloplasma mollicute	
M1c	90	*Wandonia haliotis* Haldis	Flavobateria
M2a	95	*Maribius salinus*	α-proteobacteria
M2b	98	*Donghicola eburneus*	α-proteobacteria
		h 12	
M2a	84	Uncultured bacterium clone	
M3a	97	Flavobacteriaceae bacterium	
M3b	97	Flavobacteriaceae bacterium	
M3c	97	Flavobacteriaceae bacterium	
M3d	97	*Photobacterium damselae* subs. *piscicida*	γ- proteobacteria
		h 48	
M2a	94	*Thalassobius gelatinovorus*	α-proteobacteria
M2c	93	*Planktotalea frisia*	α-proteobacteria
M2d	98	*Ruegeria lacuscaerulensis*	α-proteobacteria
M2h	99	Unidentified alpha proteobacterium	
M2i	99	Uncultured bacterium clone	
		Alibio	
		h 0	
A1a	90	*Wandonia haliotis* Haldis	Flavobacteria
A1c	90	*Cytophaga fermentans*	Cytophagaceae
A1d	90	*Sebaldella termitidis*	
A4a	90	*Wandonia haliotis* Haldis	Flavobacteria
A4b	90	*Cytophaga fermentans*	Cytophagaceae
A4c	96	*Photobacterium damselae* subsp. *piscicida*	γ-proteobacteria
		h 12	
A1a	97	Flavobacteriaceae bacterium	
A1b	96	Flavobacteriaceae bacterium	
A1c	89	Uncultured bacterium clone	
A1d	90	*Cytophaga fermentans*	Cytophagaceae
A1e	97	Flavobacteriaceae bacterium	
A1f	98	*Photobacterium damselae* sub sp. Piscicida	γ-proteobacteria
A1g	89	Uncultured bacterium clone	
A1i	90	Uncultured bacterium	
A1j	87	*Vibrio furnissii*	γ-proteobacteria
A3a	85	Desulfovibrionaceae bacterium	
A3b	93	*Wandonia haliotis* Haldis	Flavobacteria
A3c	98	*Ruegeria lacuscaerulensis*	α-proteobacteria
A4a	90	*Wandonia haliotis* Haldis	Flavobacteria
		h 48	
A1b	92	Flavobacteriaceae bacterium	
A2a	90	*Paracoccus versatus*	
A2b	82	*Vibrio* sp.	γ-proteobacteria
A2c	91	Uncultured bacterium clone	
A2d	100	Donglicola eburneus	α-proteobacteria
A3b	90	*Wandonia haliotis* Haldis	Flavobacteria
A3c	92	Flavobacteriaceae bacterium	
A3d	98	*Ruegeria lacuscaerulensis*	α-proteobacteria
A3e	98	Uncultured bacterium clone	

Continuation

OTU	SIM (%)	Closest relative	Phylogenetic group
		Negative control	
		h 0	
C2b	97	Flavobacteriaceae bacterium	
C2c	99	*Photobacterium damselae* subsp. *piscicida*	γ-proteobacteria
		h 12	
C1a	89	Flavobacteriaceae bacterium	
C1b	85	Alpha proteobacterium	
C2a	91	*Wandonia haliotis* Haldis	Flavobacteria
C2b	97	Flavobacteriaceae bacterium	
C2c	89	*Sebaldella termitidis*	Fusobacteria
C2f	96	Uncultured bacterium clone	
C2g	89	*Elizabethkingia anophelis*	Flavobacteria
C3a	99	*Thalassobius* sp.	α-proteobacteria
C3b	93	*Thioprofundum lithotrophica*	γ-proteobacteria
C3c	97	*Ruegeria lacuscaerulensis*	α-proteobacteria
C3f	91	Uncultured bacterium clone	
		h 48	
C1a	92	*Vibrio mediterranei*	γ-proteobacteria
C1b	98	*Ruegeria lacuscaerilensis*	α-proteobacteria
C1c	99	Uncultured bacterium clone	
C2a	95	*Oceanicola* sp.	α-proteobacteria
C2b	91	Flavobacteriaceae bacterium	
C2c	97	*Ruegeria lacuscaerulensis*	
C2d	92	*Vibrio* sp.	γ-proteobacteria
C2e	97	Uncultured bacterium clone	
C3a	97	*Thalassobius mediterraneus*	α-proteobacteria
C3b	92	*Thioclava pacifica*	
C3c	97	*Ruegeria lacuscaerulensis*	α-proteobacteria
C3d	87	Uncultured bacterium clone	
		Positive control	
		h 0	
C2c	99	Uncultured bacterium clone	
C3b	97	Uncultured bacterium clone	
C3c	94	Flavobacteriaceae bacterium	
		h 12	
C1a	90	*Wandonia haliotis* Haldis	Flavobacteria
C1b	97	*Ruegeria lacuscaerulensis*	α-proteobacteria
C1c	87	Uncultured bacterium clone	
C1d	97	*Maribius salinus*	α-proteobacteria
C2a	93	Uncultured bacterium clone	
C2b	87	Uncultured bacterium clone	
C2c	95	*Thalassobius* sp.	α-proteobacteria
C3a	87	Uncultured bacterium clone	
C3b	88	Uncultured bacterium clone	
C3c	90	*Donghicola eburneus*	α-proteobacteria
T48			
C2a	91	Uncultured bacterium clone	
C2b	100	*Donghicola* sp.	α-proteobacteria
C2c	87	Uncultured bacterium clone	
C3a	98	Uncultured bacterium clone	
C3b	90	*Wandonia haliotis* Haldis	Flavobacteria
C3c	98	*Ruegeria lacuscaerulensis*	α-proteobacteria
C3d	98	*Vibrio parahaemolyticus*	γ-proteobacteria
C3e	92	*Vibrio* sp.	γ-proteobacteria
C3h	99	Uncultured bacterium clone	

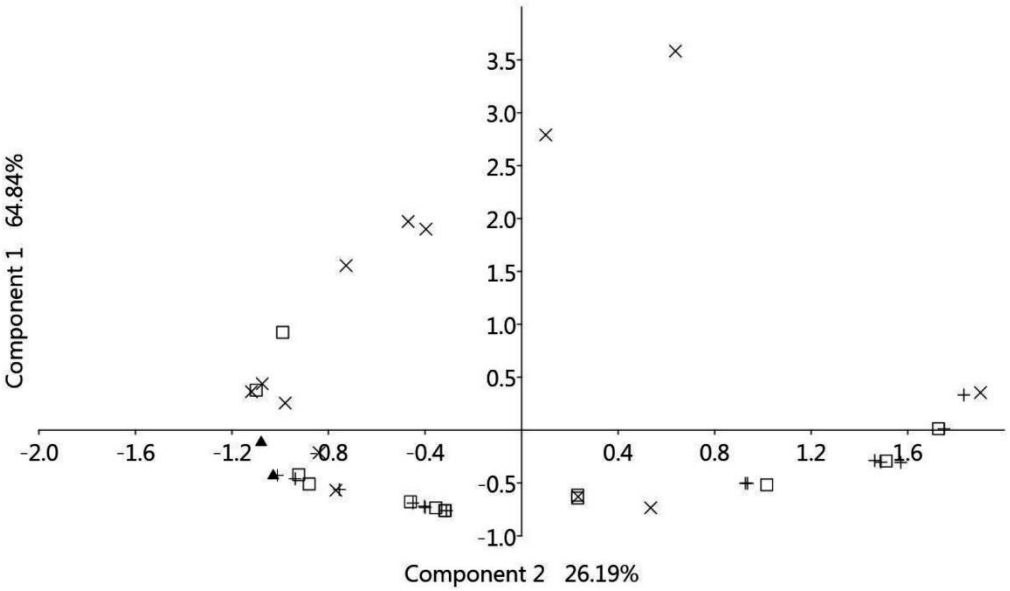

Figure 2. Principal components analysis using the Dice coefficient of single strand conformation SSCP profiles associated with the intestines of individual *L. vannamei* inoculated with the treatments and challenged with *V. parahaemolyticus* for each time: Time start (▲), 0 h (Δ), 12 h (□), 48 h (×). Each point represents a SSCP profile from one shrimp.

Recent studies on the modulation and stabilization of gut microbiota by probiotic treatment suggest that probiotics can exert a positive effect on uncultivable gut microbiota (Sáenz de Rodrigáñez *et al.*, 2009; Sun *et al.*, 2012a, 2012b; Yang *et al.*, 2012), which coincides with the modification of the gut microflora and the increase in bacterial diversity after probiotic administration reported in *Solea senegalensis* by Tapia-Paniagua *et al.* (2010). Several studies indicated that the culturable intestinal microbial community of shrimp was mainly composed of *Aeromonas*, *Plesiomonas*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio* species (Moss *et al.*, 2000; Oxley *et al.*, 2002). Of these, only *Photobacterium and Pseudoalteromonas* were detected in this work whereas *Vibrio* species were detected only in the positive control (*V. parahaemolyticus*, confirming the induced infection, and *Vibrio* sp.), and in shrimps treated with Alibio (*Vibrio* sp.), possibly because several *Vibrio* or *Vibrio*-related species are common in commercial probiotic mixtures (Verschuere *et al.*, 2000; Qi *et al.*, 2009**).**

In our case, the indigenous intestinal microbiota tended to be dominated by *Wandonia haliotis* Haldis, which may be considered a commensal, because it was present at all times and in all treatments. The presence of *V. mediterranei* in the negative control may be due to its occurrence in natural microbiota, because this species is commonly associated to a wide-range of hosts, with mutual interactions which may range from mutualism or symbiosis to a pathogenic relation (Turner *et al.*, 2009; Senderovich *et al.*, 2010).

The fact that shrimps treated with the *Bacillus* mix did not show the presence of the pathogen injected may be taken as an indication of a protective effect of this probiotic, similar to the effect against *V. parahaemolyticus* of the indigenous intestinal microbiota modified with a *Bacillus*-based probiotic observed by Wu *et al.* (2014) in the mud crab *S. paramamosain*. Modifications of the intestinal microflora by a probiotic *Bacillus* resulting in inhibition of growth of intestinal *Vibrio* spp. have been reported also in *Penaeus monodon* by Rengpipat *et al.* (2000) and by Vaseeharan & Ramasamy (2003), who also noted a positive effect in the external water environment.

Photobacterium damselae subsp. *piscicida* was observed in all our treatments. This microorganism (formerly *Pasteurella piscicida*) is a highly pathogenic bacterium that causes photobacteriosis and does not show host specificity (Toranzo *et al.*, 1991; Noya *et al.*, 1995) but, in spite of its generalized presence it did not show any pathogenic effect, possibly because the presence of the probiotic *Bacillus* strains, since these are known to modulate shrimp gut bacterial communities (Luis-Villaseñor *et al.*, 2013), thereby improving their immune response against pathogenic bacteria (Zokaeifar *et al.*, 2012, 2014).

The effect of probiotic protection on the structure of the intestinal bacterial community of shrimp infected with pathogenic bacteria was unknown, and this work

shows that both probiotic mixtures, Alibio and *Bacillus* mix, helped to maintain a natural balance in the bacterial community of the shrimps intestine, modulating and increasing diversity and evenness of bacterial species in shrimps challenged by bacterial infection.

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