Probiotics in the intestinal tract of juvenile whiteleg shrimp

**Litopenaeus vannamei**: modulation of the bacterial community

Irasema E. Luis-Villaseñor · Thelma Castellanos-Cervantes · Bruno Gomez-Gil · Ángel E. Carrillo-García · Ángel I. Campa-Córdova · Felipe Ascencio

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**Abstract** Molecular analysis of the 16S rDNA of the intestinal microbiota of whiteleg shrimp *Litopenaeus vannamei* was examined to investigate the effect of a Bacillus mix (*Bacillus endophyticus* YC3-b, *Bacillus endophyticus* C2-2, *Bacillus tequilensis* YC5-2) and the commercial probiotic (Alibio®) on intestinal bacterial communities and resistance to *Vibrio* infection. PCR and single strain conformation polymorphism (SSCP) analyses were then performed on DNA extracted directly from guts. Injection of shrimp with *V. parahaemolyticus* at 2.5 \( \times \) 10^5 CFU g^{-1} per shrimp followed 168 h after inoculation with Bacillus mix or the Alibio probiotic or the positive control. Diversity analyses showed that the bacterial community resulting from the Bacillus mix had the highest diversity and evenness and the bacterial community of the control had the lowest diversity. The bacterial community treated with probiotics mainly consisted of \( \alpha - \) and \( \gamma - \) proteobacteria, fusobacteria, sphingobacteria, and flavobacteria, while the control mainly consisted of \( \alpha - \) proteobacteria and flavobacteria. Differences were grouped using principal component analyses of PCR-SSCP of the microbiota, according to the time of inoculation. In *Vibrio parahaemolyticus*-infected shrimp, the Bacillus mix (~33 %) induced a significant increase in survival compared to Alibio (~21 %) and the control (~9 %). We conclude that administration of the Bacillus mix induced modulation of the intestinal microbiota of *L. vannamei* and increased its resistance to *V. parahaemolyticus*.

**Keywords** Whiteleg shrimp · Bacteria community · Probiotics · Bacillus mix

**Introduction**

Despite rapid expansion, aquaculture production has been negatively affected by infectious diseases, including viruses, rickettsiae, bacteria, fungi, and parasites, causing billions of dollars in losses (Lightner 2005). Increasing demand for aquaculture products has led to indiscriminate use of chemicals and antibiotics, resulting in environmental degradation (Bachére 2000). One alternative includes adding probiotics during rearing, either in the water or as feed supplements (Moriarty 1998). Among available probiotics, *Bacillus* bacteria are widely used because it produces spores that are extremely resistant to external physical and chemical processes and produce polypeptides (bacitracin, gramicidin S, polymyxin, and tyrothricin) that are active against a broad range of Gram positive and negative bacteria, including pathogenic vibrios (Morikawa et al. 1992; Perez et al. 1993; Drablos et al. 1999).

Composition of aquatic bacterial communities in ponds has a strong influence on the internal bacterial flora of farmed marine animals; this is vital for their nutrition, immunity, and disease resistance (Luo et al. 2006). At the same time, it impacts and is impacted by the bacterial

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communities in nearby marine environments that receive production effluents (Guo and Xu 1994). Intestinal micro-
biota of cultivated aquatic organisms is an important factor in maintaining health, either by preventing colonization by pathogens, decomposition of food, production of antimicrobial compounds, releasing nutrients, and maintaining normal mucosal immunity (Escobar-Briones et al. 2006).

To analyze the composition and changes of intestinal microbiota, culture-dependent methods are considered inadequate because more those 99 % of all bacteria cannot be cultivated (Amann et al. 1995). Single-strand conformation polymorphism (SSCP) is based on sequence-specific separation of the polymerase chain reaction (PCR)-derived rRNA gene amplicons in polyacrylamide gels to study the diversity of microbes, based on sequence differences of PCR products of the 16S rDNA gene amplified from different microbes (Dohrmann and Tebbe 2004). Many studies have reported the effects of probiotic treatments in aquaculture (Chythanya et al. 2007; Farzanfar 2006). There are reports on the beneficial effects of probiotics in shrimp health (Ziaei et al. 2006; Rodriguez et al. 2007). An understanding on the effects of probiotics on the indigenous gut microbiota is necessary to understand the underlying mechanisms that lead to host benefits. Similar studies have been performed in vertebrates (Escobar-Briones et al. 2006; Tapia-Paniagua et al. 2010); however, studies of invertebrates are limited. They include Pacific whiteleg shrimp Litopenaeus vannamei (Johnson et al. 2008), Kuruma shrimp Marsupenaeus japonicus (Liu et al. 2010), European lobster Homarus gammarus (Daniels et al. 2010), and Chinese fleshy prawn Fenneropenaeus chinensis (Liu et al. 2011).

The purpose of this study was to assay the composition of bacterial communities in juvenile whiteleg shrimp L. vannamei to determine the impact of probiotics in modulating intestinal microbiota. The selection of probiotics was based on their in vitro antagonism to pathogenic bacteria, hemolytic activity, adhesion to shrimp intestinal mucus (important factor in colonizing and remaining in the gut for a moderate length of time), and their effect on survival and metamorphosis of shrimp larvae.

Materials and methods

Probiotic strains

A probiotics mix of Bacillus tequilensis YC5-2, B. endo-
phyticus C2-2, and B. endophytyticus YC3-B was thawed in an ice bath. These strains were selected for known high antagonistic activity against pathogenic Vibrio strains and strong adhesion to shrimp intestinal mucosa, as well as improved survival of shrimp larvae (Luis-Villaseñor et al. 2011). Bacteria were grown in 200 mL Erlenmayer flasks with 100 mL TSB at 37 °C for 24 h. The cultures were centrifuged at 5,000 xg for 10 min; each pellet was re-suspended in a sterile saline solution containing 3 % (w/v) NaCl (S-7653, Sigma, St. Louis, MO). Absorbance was adjusted to optical density of 1 at 600 nm, approximately 1 x 10^9 cells mL^-1. These probiotics were added in the rearing system at a final concentration of 0.1 x 10^6 cells mL^-1.

Pathogenic bacterium

The shrimp bacteria pathogen Vibrio paraheamolyticus CAIM 170 was obtained from the Colección de Microorganismos de Importancia Acuicola (CAIM). The strain was maintained in trypticase soy broth (#236950, Difco, Franklin Lakes, NJ) containing 3 % (w/v) NaCl and 15 % (v/v) glycerol at −80 °C until used. The culture was cen-
trifuged at 5000 xg for 10 min; the pellet was re-sus-
pended in 3 % (w/v) sterile saline solution. The density of bacteria was adjusted to 1.0 to obtain a final density of 1 x 10^9 cell mL^-1; the inoculum was serially diluted to a density of 0.1 x 10^9 cells mL^-1.

Probiotic treatment and infection

Pacific whiteleg shrimp were obtained from a commercial hatchery. The shrimp were acclimated from five days in three tanks to laboratory conditions (filtered seawater at 28 °C and salinity of 36 ppt) before the start of the trial. There were three treatment groups, each containing 21 juvenile shrimp (mean: 8 ± 1 g) raised in 80 L tanks. The first was treated with 0.1 x 10^9 CFU mL^-1 of the Bacillus mix. for 20 days. The second group was treated with 1 x 10^6 CFU mL^-1 of the commercial probiotic Alibio (AliBio, Mexico City). The third group served as the control group and fed with a regular diet during the entire trial. Salinity of the seawater was 36 ppt and was kept at 29 °C. Continuous aeration was maintained in all tanks and 50 % of the water was changed daily. Groups 1 and 2 were maintained in five tanks; Group 3 was maintained in six tanks (three positive and three negative).

Challenge trials were carried out in shrimp after 168 h treatment with probiotics injected into the fifth abdominal section at the volume of 20 µL of Vibrio suspension (=1 x 10^8 CFU mL^-1), resulting in 2.5 x 10^5 CFU g^-1. Positive controls were injected with the same dose of Vibrio. Negative (unchallenged) controls were injected with sterile saline solution. Accumulated mortality of the shrimp was recorded.

Sample collection and DNA extraction

Shrimp were sampled at 5 day intervals, starting with exposure to probiotics, where one shrimp from each tank
was tested, five shrimp in total for each treatment. The body surface of the shrimp was washed and disinfected with 70 % ethanol before removing the entire intestinal tract. Each whole gut sample was excised with sterile forceps and scissors and placed in 1 mL absolute ethanol to precipitate DNA. Each gut was transferred to a screwed-capped tube and stored at −80 °C. To assay for diversity of the intestinal communities, chromosomal DNA was extracted (Wizard genomic DNA purification kit, Promega, Madison, WI), according to the manufacturer’s instructions.

Amplification of 16S rRNA

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 is 5’-CAGCAGCGCCGGTAAATAC and Com2ph is 3’-CCGTC AATTCTTTGAGTTT (Schwiger and Tebbe 1998). Each PCR was performed in a total volume of 50 μL in 0.2 mM micro tubes. Reaction mixtures were contained in 1 × PCR buffer with 1.5 mM MgCl₂, 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 and thermocycling (Peltier Thermal Cycler, Bio-Rad Laboratories, Hercules, CA), starting with an initial denaturation for 3 min at 94 °C, then 30 cycles for 60 s at 94 °C, then one cycle for 60 s at 53 °C and one for 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 (Schwiger and Tebbe 1998) was used for profiling bacterial communities. All PCR products of each replicate were purified (PCR purification kit, Qiagen, Hilden, Germany). The purified products was confirmed on 1 % (w/v) agarose gel. The PCR-amplified products were sequenced by a commercial firm (Genewiz, South Plainfield, NJ).

Electrophoresis was carried out for 18 h at 260 V at 20 °C (DCode Universal Mutation System, Bio-Rad Laboratories, Hercules, CA). After completion of electrophoresis, the gel was stained with AgNO₃ (Benbouza et al. 2006) and scanned using Power Look III (Umax Systems, Willich, Germany).

Analysis of SSCP profiles

Gel analysis software (GelCompar II, Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate similarities between profiles of bacteria from the different treatments and times of inoculation. These images were visualized with bacteria markers (Bacillus licheniformis, Rhizobium trifolii, Flavobacterium johnsoniae, and Rhizobium radiobacter). 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 et al. 2001), resuspended in 12 μL Tris buffer (10 mMTris-HCl, pH 8.0), and amplified by PCR, using primers Com1 and Com2ph under the conditions described above. PCR-amplified products were sequenced by a commercial firm (Genewiz, South Plainfield, NJ).

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

Statistical analysis

To determine the structural diversity of the microbial community corresponding to the SSCP banding pattern, two indices were calculated: (1) Shannon’s diversity index: \( H' = \sum [p_i \ln p_i] \), which reflects the diversity of the whole microbial community and (2) Shannon’s equitability index \( E' = H'/\ln(S) \), where S is the number of observed operational taxonomic units (presumed species) and \( p_i \) is the proportion of the intensity band (Dethlefsen et al. 2008). To determine the similarity between treatments, data of densitometric curves obtained from each sample were exported as a binary matrix (PAST software, palaeo-
electronica.org). PCA was performed from correlation matrices generated from a binary matrix, which was expressed as a value of Pearson’s similarity coefficient (Fromin et al. 2002). PCA analysis was conducted with software (Statistica 6.0, StatSoft, Tulsa, OK).

Results

Analysis profiles from SSCP

SSCP analysis, using universal primers, targeted the V4 and V5 regions of the 16S rRNA gene to estimate bacterial diversity and identify the dominant intestinal bacteria in juvenile whiteleg shrimp. The fingerprints demonstrate that probiotic replicates were the most closely related to the native intestinal microbiota, which clustered into a group distinctly different from the profiles in the control groups (Fig. 1a). From four SSCP gels, 119 bands were sequenced and identified.

At day 5, bacterial patterns from gut samples inoculated with probiotics showed uniformity in the composition of the microbiota and clustering with high similarity 71 % for the *Bacillus* mix treatment and 81 % for the Alibio treatment. Both had higher similarity than the control group with 31 % (Fig. 1a).

The dendrogram analysis at day 10 showed that the SSCP pattern in samples from shrimp treated with the *Bacillus* mix were clustered into one group: 62 % for M1–M2 and 83 % for M4–M5. Shrimp treated with Alibio were clustered into a different group with similarity of 73 % (A1–A5). Results were heterogeneous in the control group, with similarity of 51 % for C1–C4 and 85 % for C2–A4 (Fig. 1b). Similarity at day 15 had the highest homogeneity between treatments: 87 % for the *Bacillus* mix M1–M3 treatments and 93 % for the M2–M4 treatments. Alibio had 88 % for A1–A3 and 94 % for A1–A5 (Fig. 2a). Similar banding patterns occurred at day 20, reaching 90 % to 99 %. Variations in the communities within each treatment group did not vary greatly (Fig. 2b).

Fig. 1 Acrylamide gel-generated by single strand conformation polymorphism (SSCP) dendrogram illustrating the relationship (percent similarity) between bacterial communities in gut of shrimp at day 5 (a) and day 10 (b) inoculated with probiotics; M1–M5 (*Bacillus* mix), A1–A5 (commercial probiotic), C1–C4 (without probiotics). Scale of dendrogram shows percent of similarity of clusters. The dendrogram was calculated with UPGMA and Pearson correlation.
The Shannon Index ($H'$) at day 5 was similar in all groups, but somewhat lower in the Alibio treatment (2.24) and control group (2.37), compared to the Bacillus mix treatment (2.74). This may be explained by the low percentage intensity bands (relative abundance); hence, molecular approaches favor the dominant strains (Table 1). In contrast, the observed taxonomic units (S, presumed species), species richness, and Shannon’s diversity index were similar in all groups, but highest in the Bacillus mix ($H' = 2.8$) at day 15. This is in contrast to the Alibio treatment, having the highest $H'$ at day 10 (2.74). Based on SSCP data at day 20, $H'$ was 2.3–2.6, a very narrow range, indicating similar diversity of species in all samples (Table 1).

Significant differences in the intestinal bacterial communities at days 5, 10, 15, and 20 were also studied, using PCA statistical methods (Fig. 3). The two principal components (PC) explained a high percentage of the total variation, PC1 was 64.5 % and PC2 was 18.5 % (82.9 % cumulative variance).

**Bacteria community structure**

Comparison of identity of each band in the SSCP profile of the samples with sequences in the NCBI database revealed ~99 % similarity with the 16S rRNA gene sequences of several strains (Table 2, ESM). Sequence analysis showed that at day 5, the shrimp gut was dominated by flavobacteria and $\alpha$-proteobacteria, represented by Wandonia halidisi-1 and Maribius salinus in the three treatments (Fig. 4). Donghicola eburneus was present only in the Bacillus mix and the control. Rhoovulum sulfidophilum was present only in the Alibio treatment, and Kordia algicida and Loktanella hongkongensis only occurred in the Control group (Table 2, ESM).
At day 10, the community was dominated by flavobacteria, fusobacteria, \( \alpha \)-proteobacteria, and \( \gamma \)-proteobacteria (Fig. 4). Four bacteria were found in the Bacillus mix treatment (Thalassobium gelatinovorus, Flavobacterium sp., Sebaldea termitidis, and Propionogenium maris). In the Alibio treatment, there were several changes, with reduction or disappearance of Maribius pelagicus and Rhodovalum sulfidophilum and higher amounts of Thalassobium mediterraneus, Thioprofundum lithotrophicum, Lutimaribacter saemakumensis, Donghicola eburneus, Tamiana crutina, and Roseobacter sp. The control group lost Loktanella honkongensis and Kordia algicida, which was replaced by Ruegeria laccuscarilensis and Thioprofundum lithotrophicum (Table 2, ESM).

At day 15, the Bacillus mix group had small populations of \( \alpha \)-proteobacteria and flavobacteria, represented by Ruegeria laccuscarilensis, Octadecabacter arcticus, Loktanella frxyellensis, and Meridianimaribacter flavus. The Alibio group had sphingobacteria and fusobacteria. Shimia marina and Rhodovalum iodosum appeared in the control group (Fig. 4).

At day 20, \( \alpha \)- and \( \gamma \)-proteobacteria, sphingobacteria, and flavobacteria were present, with few variations between treatments. The Bacillus mix group was dominated by \( \gamma \)-proteobacteria, the Alibio treatment by sphingobacteria, and both phylogenetic groups were absent in the control group (Fig. 4). Resident populations of intestinal bacteria in all treatments and at all sampling times included Maribius salinus and Donghicola eburneus (\( \alpha \)-proteobacteria) and Wandonia haliotis (flavobacteria) (Table 2).

### Survival of shrimp

To determine whether probiotics protect shrimp against vibriosis, shrimp were infected with *V. parahaemolyticus* by injection. The final survival of shrimp treated with the Bacillus mix was 33.3 %; Alibio was 21.1 %; untreated shrimp had 9.5 % survival (Fig. 5). Statistical analysis indicate significant differences \((P < 0.05)\) in mortality between the Bacillus mix group and the control group.

### Discussion

Analysis of gut samples obtained from PCR-SSCP indicate that the gut microbiota of the shrimp has limited bacterial diversity and that, after exposure for 10 days to the probiotics, there were significant changes in the intestinal microbiota of shrimp from the mix of three *Bacillus* strains. The SSCP profiles of shrimp gut populations exposed to the Bacillus mix and Alibio were very similar among replicates. Analysis of the SSCP fingerprints confirmed that the composition of the intestinal microbiota of shrimp exposed to the Bacillus mix was significantly different than the controls. In our study, most of the OTUs (operational taxonomic unit) identified by SSCP gels treated with the probiotics belong to phylogenic groups class \( \alpha \)- and \( \gamma \)-proteobacteria, flavobacteria, shingobacteria, and fusobacteria, compared with other species of invertebrates, where the microbiota were represented by class \( \alpha \)-, \( \gamma \)-, and \( \varepsilon \)-proteobacteria in the Chinese fleshy prawn *Fenneropenaeus chinensis* (Liu et al. 2011), by fusobacteria and \( \gamma \)-proteobacteria in the giant tiger prawn *Peneaus monodon* (Chaiyapechara et al. 2011), and by derribacteria, mollicutes, \( \gamma \)- and \( \varepsilon \)-proteobacteria, small fractions of firmicutes, cytophaga-flavobacter-bacteroides, verricibacteriae, and \( \beta \)- and \( \delta \)-proteobacteria in vent shrimp (Durand et al. 2010). Furthermore, the gut content of shrimp exposed to the Bacillus mix and Alibio had higher bacterial diversity, compared with the controls, supported by the total number of OTUs, high Shannon diversity (\( H' \)), and evenness (\( E' \)).

Intestinal bacterial communities were dominated by \( \alpha \)-proteobacteria and flavobacteria at all times in shrimp treated with probiotics. The resident community included *Maribius salinus* and *Donghicola eburneus* (\( \alpha \)-proteobacteria) and *Wandonia haliotis* (flavobacteria) in all treatments. Dominance of \( \gamma \)-proteobacteria occurs in intestinal communities of other crustaceans, including *Fenneropenaeus chinensis* (Liu et al. 2011), ornate rock lobster *Panulirus ornatus* (Payne et al. 2007), and European lobster *Homarus gammarus*.
L. (Daniels et al. 2010), and Penaeus monodon (Chaiyapechara et al. 2011). Dempsey et al. (1989) suggest that only one or two phylogenic groups dominate the shrimp gut and have very low diversity; reports of gut communities in shrimp were based mainly on culture-dependent, microbiological techniques. Comparisons with molecular techniques indicate that 10–50 % of the population is cultivable (Holzapfel et al. 1988). Since the SSCP monitors the predominant bacteria in a sample, bands representing Bacillus probionts were not detected because the density of probiotic strains is $<0.1 \times 10^6$ CFU mL$^{-1}$. Gelsomino et al. (1999) report that only abundant species generate a band because the detection limit for bacteria in DGGE may be as high as $10^6$ cells and the PCR-DGGE system will generally detect a limited number of dominant, ubiquitous, and ecologically recalcitrant bacterial types.

At day 5, sequences obtained indicate a modulation of intestinal microbiota treated with the Bacillus mix was a highly diverse community. At day 10, the sequences display the effect of probiotics in modulating intestinal microbiota, similar to the findings of Liu et al. (2010), where Bacillus spp. in feed for Marsupenaeus japonicas increased individual variation and diversity of species. Microbial communities at days 15 and 20 were more stable, with few variations and greater similarity. Similar results were obtained by Daniels et al. (2010), reporting that mannan oligosaccharides and Bacillus stabilize the indigenous microbial populations in European lobster Homarus gammarus and, to some extent, suppresses variations and influx of new microbial species from the rearing environment.

![Figure 3](image1.png)

**Fig. 3** Principal components analysis using Pearson correlation of single strand conformation polymorphism (SSCP) profiles associated with the intestines of individual Litopenaeus vannamei inoculated with the treatments for each time: day 5 (+), day 10 (open square), day 15 (×), and day days (*). Each point represents a SSCP profile from one shrimp.

![Figure 4](image2.png)

**Fig. 4** Percent composition of intestinal bacterial community of individual L. vannamei inoculated with probiotics Bacillus mix (M5–M20), Alibio (A5–A20), and Control (C5–C20), based on 16S rRNA; result by percent composition

![Figure 5](image3.png)

**Fig. 5** Percentage survival of juvenile shrimp Litopenaeus vannamei over a 48 h period challenge to Vibrio parahaemolyticus $2.5 \times 10^5$ CFU g$^{-1}$ shrimp or sterile 3 % NaCl solution. *Significantly different than control.
In our study; the *Bacillus* mix and Alibio increased similarity in the microbial communities over time, compared to the control group. The *Bacillus* mix and Alibio treatments that modulated microbiota and reduced diversity of gut communities also influenced the health of the host. Highest survival to the challenge from *Vibrio parahaemolyticus* occurred with shrimp treated with *Bacillus* mix (~33 %), followed by Alibio (~21 %); the control group had little resistance to the challenge (~9 %). Other reports indicating benefits include Lau et al. (2002), where the gut bacteria *Cytophaga* spp. provides short chain fatty acids and attaches to surfaces and exhibit strong chitinase and cellulose activities in the bay ghost shrimp *Neotrypaea californiensis* and Liu et al. (2009), where *B. subtilis* E20 contributes more protease to the gut of whiteleg shrimp or stimulates production of endogenous proteases.

In summary, we found γ-proteobacteria, fusobacteria, sphyngobacteria, and flavobacteria to be the predominant bacterial types in the intestine of whiteleg shrimp *Litopenaeus vannamei*. Furthermore, SSCP fingerprints demonstrated that the composition of the intestinal microbiota of shrimp treated with the *Bacillus* mix was distinctly different from the control group. The addition of the *Bacillus* mix significantly reduced species diversity and richness and increased similarity of the microbial types. The study contributes to a better understanding of the composition and dynamics of shrimp gut microbiota and to the development of a *Bacillus* mix with probiotic properties for shrimp cultivation.

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