Beneficial effects of four Bacillus strains on the larval cultivation of Litopenaeus vannamei

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A B S T R A C T
We studied the effects of four strains of Bacillus isolated from the guts of healthy wild adult shrimp on survival and rate of development of whiteleg shrimp Litopenaeus vannamei larvae to understand how endemic Bacillus probiotic strains improve the health of larvae. This included bacterial isolation, assays of hemolytic activity, antagonism against pathogenic Vibrio species, growth, and adhesion to shrimp intestinal mucus, and molecular identification. Bacillus strains were tested on larval shrimp using a daily concentration of $1 \times 10^5$ CFU mL$^{-1}$, starting each bioassay at nauplii V at a density of 225 nauplii L$^{-1}$. Inoculations of four natural, commercial products and antibiotic oxytetracycline were added directly to the water. All treatments induced a significant increase in survival compared to the control, with strain YC5-2 showing the highest survival (~67%), followed by AlibioMR (~57%). A mix of two strains induced the highest rate of development (7.00), followed by AlibioMR (6.35). The results showed remarkable antagonistic activity by the four non-hemolytic Bacillus strains against Vibrio campbelli, V. vulnificus, V. parahaemolyticus, and V. alginolyticus and the potential and efficiency of probiotics isolated from shrimp gut to improve survival of shrimp larvae.

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1. Introduction

Intensive shrimp culture practices involve an indiscriminate use of chemicals and antibiotics to prevent mortality of larvae and juvenile marine species (Peraza-Gómez et al., 2009). Probiotic organisms are an alternative to antibiotics because antibiotics have led to resistance of bacterial pathogens (Gatesoupe, 1999). Previous applications of probiotic organisms have shown beneficial host effects, including improved growth, survival, and health (Moriarty, 1998; Skjermo and Vadstein, 1999). Various mechanisms have been proposed to explain their beneficial effects, including competition for adhesion sites, competition for nutrients, enzymatic contribution to digestion, improved water quality, and stimulation of the host immune response (Kumar-Sahu et al., 2008). Selection of probiotics in aquacultural enterprises is usually based on results of tests showing antagonism toward the pathogens, an ability to survive and colonize the intestine, and a capacity to increase an immune response in the host.

Previous studies show that inoculation with a probiotic strain during cultivation of whiteleg shrimp Litopenaeus vannamei larvae (nauplii stage V) prevents colonization by a pathogenic strain, because the probiotic succeeds in colonizing the gut of the larvae (Gómez-Gil et al., 2000; Zherdmant et al., 1997). Nevertheless, studies of probiotics to improve growth or survival in crustacean larvae are scarce. Recently, methods for improving water quality of hatcheries and application of probiotics has gained momentum (Balcázar et al., 2007a; Gómez et al., 2008; Guo et al., 2009; Van Hai et al., 2009). Daily administration of probiotics based on Bacillus spp. during hatchery and farming stages leads to higher feed conversion ratios, improved specific growth rates, and higher final shrimp biomass than controls (Guo et al., 2009; Liu et al., 2009a). Metamorphosis improved with administration of the probiotic B. fusiformis (Guo, et al., 2009). Zhou et al. (2009) found that B. coagulans SC8168, as a water additive at certain concentrations, significantly increased survival and some digestive enzyme activities of shrimp larvae. Bacillus spp. possesses adhesion abilities, produce bacteriocins, and provide immunostimulation (Ravi et al., 2007).

Nguyen et al. (2007) suggest that the beneficial effect of the probiotics on the host has been wrongly attributed to what is found during in vitro observations, that in vivo physiology might be different from in vitro metabolic processes. Development of suitable probiotics is not a simple task and requires full-scale trials, as well as development of appropriate monitoring tools and controlled production (Decamp et al., 2008). In vitro and in vivo studies are needed to demonstrate antagonisms to pathogens and their effect on survival and growth of the host.

Bacillus spp. is often used as benefic microorganisms in aquaculture systems (Nakayama et al., 2009). However, the mechanism of
action is not completely understood. In this study, the effect of probiotics isolated from _L. vannamei_, two commercial probiotics, and a commercial antibiotic were evaluated on survival and development of shrimp larvae. Additionally, the capacity to adhere and grow on the intestinal mucosa were studied to understand how endemic probiotics improve health in larvae.

2. Materials and methods

2.1. Bacterial isolates

Bacterial strains were isolated from the intestinal tract of adult shrimp. The contents of the foregut (stomodeum) were aseptically removed from live, healthy shrimp. The samples were homogenized and serially diluted, plated on marine agar (#2216, Difco; Becton, Dickenson and Company, Franklin Lakes, NJ) and incubated at 37 °C for 24–48 h. Colonies of single, dominant types were selected and re-streaked onto marine agar to obtain pure cultures after incubation for 24 h at 37 °C; 20 colonies from the intestine were also characterized and selected if determined to be rod-shaped and Gram-positive. Of these, nine strains were selected and stored at −85 °C in tryptic soy broth (TSB) containing 2.5% (w/v) NaCl and 15% (v/v) glycerol until used.

2.2. Hemolytic activity of _Bacillus_ strains

To measure hemolytic activity of the various _Bacillus_ strains on erythrocytes, nine isolated _Bacillus_ probiotic strains were inoculated by streaking on plates containing blood-based agar (#211728, Difco) supplemented with 5% (w/v) human sterile blood and 3% (w/v) NaCl. Plates were inoculated at 37 °C for 24 h and results were determined, as described by Koneman et al. (2001), as: α-hemolysis (slight destruction of hemocytes and erythrocytes with a green zone around the bacterial colonies); β-hemolysis (hemolysin that causes a clean hemolysis zone around the bacterial colonies); and γ-hemolysis (without any change in the agar around the bacterial colonies. Hemolytic activity in shrimp hemocytes was tested, as described by Chin-l et al. (2000). Briefly, a 1-ml syringe was rinsed with EDTA buffer (450 mmol L\(^{-1}\) NaCl, 10 mmol L\(^{-1}\) KCl, 10 mmol L\(^{-1}\) EDTA-Na\(_2\), and 10 mmol L\(^{-1}\) HEPES at pH 7.3). After disinfecting the surface of shrimp unveiling~20 g with 70% ethanol; hemolymph was drawn with a sterile needle from between the fifth pair of pereiopods; 1 ml hemolymph was immediately transferred to a sterilized tube containing 0.2 mL EDTA buffer and stained with 133 μL 3% (w/v) Rose Bengal dye (#R4507, Sigma St. Louis, MO) dissolved in EDTA buffer with gentle shaking to achieve complete mixing. Aseptically, 1 ml of the stained hemolymph preparation was added to 15 mL sterile basal agar medium containing (10 g L\(^{-1}\) Bacto peptone (#211677, Difco), 5 g L\(^{-1}\) HCl, and 15 g L\(^{-1}\) Bacto agar (#214050, Difco) at pH 6.8) cooled to 45–50 °C, followed by gentle mixing and poured into Petri dishes. Shrimp blood agar plates with a rose red color were considered satisfactory because of the homogeneously distributed stained hemocytes. When the hemocytes were destroyed by hemolytic bacteria, a clear zone (>4 mm) appeared around the colonies.

2.3. Pathogenic strains

Pathogenic bacterial strains _Vibrio harveyi_ CAIM 1793, _V. parahaemolyticus_ CAIM 170, _V. campbelli_ CAIM 333, _V. alginolyticus_ CAIM 57, and _V. vulnificus_ CAIM 157 were obtained from the Colección de Microorganismos de Importancia (CAIM, www.ciad.mx/caim). Strains were maintained in trypticase soy broth (#236950, Difco) containing 3% (w/v) NaCl and 15% (v/v) of glycerol at −80 °C until used.

2.4. Antagonism test

The nine potential probiotic strains and five pathogenic strains were thawed in an ice bath. Each of the bacterial isolates was grown in 10 mL TSB at 30 °C for 24 h. Each sample was centrifuged at 5000 g for 10 min; each pellet was suspended in a sterile saline solution containing 3% (w/v) NaCl. Density of bacteria was measured by spectrophotometry (DU 640, Beckman Coulter, Brea, CA) at 600 nm. The optical density was adjusted to 1.0 nm to obtain a final density of 1 × 10\(^7\) cells mL\(^{-1}\); this inoculum was serially diluted to a density of 1 × 10\(^5\) cells mL\(^{-1}\) for in vitro antagonistic tests, according to Dopazo et al. (1988). For this test, 10 μL of each suspension of bacteria were blotted on the surface of trypticase soy agar (TSA) + NaCl medium (TSA containing 3% (w/v) NaCl (S-7653, Sigma) and then incubated for 24 h at 37 °C. The plates were then placed in a closed chamber and exposed to chloroform vapors for 45 min to kill the bacteria. Each plate was covered with 6 mL TSA + NaCl medium containing a 0.1 mL suspension of either _V. harveyi_ CAIM 1793, _V. parahaemolyticus_ CAIM 170, _V. campbelli_ CAIM 333, _V. alginolyticus_ CAIM 57, or _V. vulnificus_ CAIM 157. Plates were examined after incubation at 30 °C for 24 h. Strains showing a >5 mm diameter inhibition halo were considered positive for the test, strains producing smaller inhibition zones were considered as sensitive without total inhibition.

An additional inhibition test was performed (Balcázar et al., 2007a), where the pathogenic strains of bacteria were inoculated in trypticase soy agar (TSA) supplemented with 2.5% (w/v) NaCl and placed in Petri dishes. Wells of 3 mm were made on solidified agar; wells were then filled with 10 μL of overnight bacterial culture. Trypticase soy broth (TSB) containing 2% (w/v) NaCl, was added as the negative control. The plates were incubated at 30 °C and zones of inhibition around the wells were measured after 24–48 h.

2.5. Adhesiveness

2.5.1. Preparation of mucin and shrimp mucus

Partially purified mucin type III derived from pig stomach (M1778, Sigma) was used, hereafter called gastric mucin. Crude mucus from the intestine of adult shrimp was obtained by gentle scraping and suspending it in cold HEPES (N-[2-hydroxyethyl]piperazine-N'-(2-ethane-sulfonic acid)) plus Hank’s balanced salt solution H-H Buffer (136.89 mmol L\(^{-1}\) NaCl, 5.37 mmol L\(^{-1}\) KCl, 1.26 mmol L\(^{-1}\) CaCl\(_2\)-2H\(_2\)O, 0.81 mmol L\(^{-1}\) MgSO\(_4\)-7H\(_2\)O, 0.35 mmol L\(^{-1}\) Na\(_2\)HPO\(_4\), 2.57 mmol L\(^{-1}\) KH\(_2\)PO\(_4\), and 9.98 mmol L\(^{-1}\) HEPES at pH 7.4). Shrimp mucus and porcine gastric mucin were conjugated with horseradish peroxidase (P8375, Sigma), as described by Hudson and Hay (1989), and stored at −20 °C.

2.5.2. Adhesion assay of shrimp mucus and porcine gastric mucin

The seven isolated strains were grown in LDM medium, described in Conway and Henriksson (1989). The adhesion assay was described by Rojas and Conway (2001). Bacteria cells were harvested, washed, and suspended in H-H buffer and adjusted to 0.9–1.0 OD at 595 nm; 10 μL of the bacterial suspensions were immobilized in Immobilon-P polyvinylidene difluoride membranes (P-15552, EMD Millipore, Billerica, MA) and a 3% bovine serum albumin (BSA) solution (AP-4500-80, SeraCare Life Sciences, Milford, MA) was used as a blocking agent at non-specific adhesion sites. The membranes were washed three times for 10 min with 10 mL H-H buffer and then incubated in 100 μL HRP-mucus or HRP-mucin (1:1000 solution) in 10 mL H-H buffer at room temperature for 2 h. Membranes were washed three times in H-H buffer (20 min each wash) and rinsed with 0.1 M sodium acetate buffer at pH 5.0 prior to development with the substrate diaminobenzidine (2.5 mg diaminobenzidine, 2.5 μL 30% hydrogen peroxide solution, and 10 mL 0.1 M sodium acetate at pH 5.0). The reaction was stopped after 5 min by rinsing the membrane in 0.1 M sodium metabisulfite.
2.5.3. Bacterial growth in mucus

Extract of shrimp intestinal mucus was sterilized for 15 min in plastic Petri dishes in a laminar flow cabinet equipped with a germicidal UV lamp (Philips TUV 30 W/G30 T8, 254 nm) (Macías et al., 2008). Sterility was tested on marine agar (#2216, Difco) and TCBS agar (#0650-17, Difco). Sterile mucus was then placed in sterile 2-mL test tubes; the mucus was inoculated with 5000 cells 200 μL–1 of mucus and incubated at 30 °C for 24 h and 48 h. Each tube of cultures was transferred to 1-mL microtubes and bacteria were collected by centrifugation. Pellets were washed with phosphate-buffer saline (PBS) (145 mmol L–1 NaCl, 2.87 mmol L–1 KH2PO4, and 6.95 mmol L–1 K2HPO4) at pH 7.2; 50 μL 0.01% acridine orange solution (A-6014, Sigma) were added to each tube. The contents were then incubated for 30 min at room temperature and washed twice with 50 μL distilled water. Wet mounts were studied by epifluorescence microscopy (Eclipse E600, Nikon, Tokyo, Japan) with a high-pressure mercury lamp and B-2A filter (EX450-490, DM505, and BA 515, Nikon) and a Plan FluorDLL100x oil F3 objective. For quantification of viable bacteria at 24 h and 48 h, cultures from each tube were diluted in H-H buffer and serial dilutions were plated on marine agar (#2216, Difco) and incubated at 37 °C for 24 h under aerobic conditions.

2.6. Molecular identification of potential probiotic strains

To identify potential probiotic strains isolated from wild shrimp, chromosomal DNA was extracted (Wizard genomic DNA purification kit, Promega, Madison, WI) according to the manufacturer’s instructions. The 16S rRNA gene was amplified (∼1.5 kb DNA) of each strain by PCR with two universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH* (5′-AAGGAGGTGATCCAGCCGCA-3′), as described by Broda et al. (1999). PCR amplification was performed in a thermocycler (Peltier, BioRad, Hercules, CA); each PCR mixture contained 1× PCR buffer (Invitrogen; Life Technologies, Carlsbad, CA), 1.5 mmol–1 MgCl2, 0.25 mmol–1 each dNTP, 0.025 μg chromosomal DNA, 1 U Taq DNA polymerase (#10342-020, Invitrogen) and 0.5 mmol–1 of each primer. Amplification included one step at 95 °C for 4 min, 35 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The presence of specific PCR products was confirmed on a 1% (w/v) agarose gel. PCR-amplified products were sequenced (Macrogen, Kumchun-Ku Seoul, ROK). The 16S rRNA sequences were identified by comparing them with the EzTaxon server database (www.eztaxon.org; Chun et al., 2007). The construction of neighbor-joining tree (see Fig. 1) and bootstrap analysis of 1000 resamplings were performed using Mega 5 software (molecular evolutionary genetics analysis, v 5.05).

2.7. Experimental broodstock

Prior to extraction of their gut, adult shrimp were maintained for two days in a 1500-L fiberglass tank containing aerated and filtered (0.2 μm) seawater containing 10 mg L–1 EDTA disodium salt. Filtered seawater was maintained at 28 °C and salinity of 36 ppt. Shrimp were fed daily with commercial pellets containing 35% protein (Grupo PIASA, Apodaca, N.L., Mexico).

For in vivo experiments, groups of 225 nauplii L–1 were raised in 40-L tanks at 29±1 °C, salinity of 36 ppt, filtered (1 μm) seawater under constant aeration. Larvae were fed 2×105 cells mL–1 Chaetoceros gracilis and Isochysis galbana (1:1) from protozoea I to III. The larval stages mysis I, mysis II, mysis III, and postlarvae were fed with heat-killed Artemia salina. The experiments were finished when larvae reached postlarvae stage. Seawater was exchanged 50% daily.

Fig. 1. Phylogenetic tree of probiotic strain based on 16S rRNA gene partial sequences, constructed with the neighbor-joining method of the MEGA program package. The bootstrap consensus tree (50% cutoff value) was constructed by using the UPGMA. Vibrio cholera was used as out group. The scale bar corresponds to 0.1 substitutions per nucleotide.
2.8. Survival and rate of development of larvae exposed to probiotic agents

The first experiment was conducted for nine days to determine the effect of the probiotic strains cultured alone or mixed in the larval culture. Groups of 225 nauplii L\(^{-1}\) were inoculated daily after exchange of water with two isolates cultured together Mix-I (YC3-b and C2-2) at 1:1 proportion and a final density of 1 \(\times 10^5\) CFU mL\(^{-1}\) (Guo et al., 2009). Additionally, strains YC5-2 and YC2-a were assayed to serve as benchmarks, as was oxytetracycline (#05750, Sigma) at 4 mg L\(^{-1}\) and two commercial probiotics, Epicin\textsuperscript{B} (Epico BioNetworks, Eastampton, NJ, USA) 1.5 mg L\(^{-1}\), and Alibio\textsuperscript{MR} (Alibio, Mexico City). Doses of Alibio\textsuperscript{MR} were adjusted to the stage of larval development: zoea I (1 mg L\(^{-1}\)), zoea II (2 mg L\(^{-1}\)), zoea III (3 mg L\(^{-1}\)), mysis (4 mg L\(^{-1}\)), and postlarvae (5 mg L\(^{-1}\)). The containers were managed in triplicate at each treatment. A control group was cultured without probiotic microorganisms or antibiotic. Survival of larvae was determined every 24 h, from nauplii until the first postlarval stage.

To observe a probable synergist or antagonistic effect between bacterial strains, measured as a percentage of survival and rate of development, we performed a second bioassay lasting nine days. Treatment 1 required inoculation of nauplii V with a mixed culture of strains YC3-b, C2-2, and YC5-2 (1:1:1) at a density of 1 \(\times 10^5\) CFU mL\(^{-1}\). In treatment 2 Alibio was added with the same changes in the levels of inoculation listed previously. The larvae were sampled to determine the effect of the potential probiotics on: (1) larval development and (2) rate of development, using the index of development (ID), described by Villegas and Kanazawa (1979); ID = \[\frac{\Sigma i}{n}\] \(i\), where \(i\) is the absolute value attributed to each larval stage (3 = ZIII; 4 = ML 5 = MII; 6 = MIII, and 7 = PL1), \(n\) is the total number of larvae at stage 1, and \(n\) is the number of organisms measured.

2.9. Accession number

Sequences of the four strains were deposited at GenBank: Strain C2-2 (HM770880), Strain YC3-b (M770881), Strain YC5-2 (HM770882), Strain YC2-a (HM770883), Strain YC1-a (HQ634208), YC3-a (HQ634209), and YC3-c (HQ698961).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) assessed for significant differences in survival (%) among treatments. Survival data were arc-sine transformed. Where significant ANOVA differences were found, Tukey's HSD test was used to identify the nature of these differences at \(P\leq0.05\). Statistical analyses were performed using Statistica v 6.0 software (StatSoft, Tulsa, OK).

3. Results

3.1. Bacterial isolates

We observed 20 morphologically different colonies on marine agar (dilutions at \(10^{-6}\) and \(10^{-7}\)). These colonies were identified, of which only nine were Gram positive, rod-shaped bacilli. These nine strains were used in the follow tests. Identification of phylogenetic neighbors was initially carried out with the BLAST program (Altschul et al., 1997) and megaBLAST program (Zhang et al., 2000) against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 50 sequences with the highest scores were selected for calculating pairwise sequence similarity using global alignment algorithm performed on the EzTaxon server (http://www.eztaxon.org; Chun et al., 2007). Analysis of 1.5-kb amplified fragments in Blast showed 98–99% identity to sequences of Bacillus species previously reported in databases (Table 1; Fig. 1).

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identity (%)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>YC3-b</td>
<td>99.52</td>
<td>Bacillus endophytics</td>
</tr>
<tr>
<td>C2-2</td>
<td>99.38</td>
<td>Bacillus endophytics</td>
</tr>
<tr>
<td>YC5-2</td>
<td>99.91</td>
<td>Bacillus tequilensis</td>
</tr>
<tr>
<td>YC2-a</td>
<td>99.59</td>
<td>Bacillus amyloliquefaciens</td>
</tr>
<tr>
<td>YC3-a</td>
<td>100</td>
<td>Bacillus licheniformis</td>
</tr>
<tr>
<td>YC1-a</td>
<td>99</td>
<td>Bacillus licheniformis</td>
</tr>
<tr>
<td>YC3-c</td>
<td>99</td>
<td>Bacillus licheniformis</td>
</tr>
</tbody>
</table>

3.2. Hemolytic activity of Bacillus strains

Four Bacillus strains (YC2-a, YC3-b, YC5-2, and C2-2) exhibited type γ hemolytic activity, three Bacillus strains (YC1-a, YC3-a, and YC3-c) exhibited type α hemolytic activity, and two Bacillus strains (YC3-d and YC2-b) having type β hemolytic activity were not assayed for hemolysis (Table 2).

3.3. Antagonism tests

The two-layer method described by Dopazo et al. (1988) shows that only two isolates (YC5-2 and YC2-a) inhibited growth of Vibrio campbelli (CAIM 333) and V. vulni (CAIM 157) with inhibition halos of 5–18 mm diameter. The well diffusion test (Balcázar et al., 2007) showed that 24-h cultures of inactivated isolates YC5-2, YC2-a, and C2-2 were able to inhibit P. paraheamolyticus (CAIM 170) and V. harvey (CAIM 1783), with inhibition halos of 11–17.5 mm diameter. V. algilamicolyticus (CAIM 57) showed sensitivity but no inhibition to these probiotic strains (see Table 2).

3.4. Mucus adhesion assay and bacterial growth in mucus

Seven strains (YC2-a, YC3-b, YC5-2, C2-2, YC1-a, YC3-a, and YC3-c) adhered to porcine gastric mucin and crude shrimp mucus (Fig. 2). The seven isolates were able to grow in the mucus 24 h after inoculation; after 48 h viable cell counts were lower (Table 3). These strains were examined for their ability to grow in shrimp intestinal mucus. Sterility of mucus was confirmed on specific media. The number of viable cells decreased by ~50% at 48 h; strains YC5-2, YC3-a, YC3-c, YC1-a, and YC2-a had viable cell counts between 1.3 \(\times 10^6\) UFC mL\(^{-1}\) and 10 \(\times 10^8\) UFC mL\(^{-1}\) at 24 h, which decreased to between 0.126 \(\times 10^6\) UFC mL\(^{-1}\) at 48 h; however, abundant free spores were observed in five strains with epifluorescence microscopy. Strains YC3-b and C2-2 had viable cell counts between 1.87 \(\times 10^6\) UFC mL and 4.14 \(\times 10^6\) UFC mL at 24 h, showing a decrease at 48 h with viable bacteria remaining about 0.18 \(\times 10^6\) UFC mL\(^{-1}\) for both strains (Fig. 3).

3.5. Probiotic effect on survival and development

Larvae inoculated with potential probiotic isolates at a density of 1 \(\times 10^6\) CFU mL\(^{-1}\) had significantly better survival than the control. The highest larval survival, compared to the control (4.9%; Fig. 4) was inoculated with isolate YC5-2 (67.3%) and the commercial probiotic Alibio\textsuperscript{TM} (57.4%).

The rate of development of larvae inoculated with a specific isolate was not significantly different from the other isolates, but all were significantly different from the control (Table 4). Based on the postteriori Tukey analysis the treatments clustered into three mutually separate groups: Group A (Control, YC2-a); Group AB (OTX, Alibio\textsuperscript{TM}, YC3-b, C2-2, and Epicin\textsuperscript{TM}); and Group B (Mix-1). There was a significant difference between Group AB and Group B compared to the control. The Mix-1 treatment produced the fastest development (ID = 6.88) and was superior to the other treatments (Table 4).
Because YC5-2 provided the highest survival rate and Mix-1 (YC3-b and C2-2) provided the fastest rate of development, these three isolates were cultured together in a second trial (Mix-2). This trial showed no significant difference in the survival rate for Mix 2 (30.5%) compared to the Alibio™ (23.5%) treatment and the control (32.8%; Fig. 5). However, the rate of development showed significant differences between Mix-2 (YC3-b, C2-2, and YC5-2) and Alibio™, compared to the control, reaching final ID values of 7.00, 6.35, and 5.76 for Mix-2, Alibio™, and the control, respectively (Table 4).

### 4. Discussion

Several species of bacteria have been used in shrimp farming as control against vibriosis, including some Bacillus, Lactobacillus, Vibrio, Streptococcus, Alteromonas, Aeromonas, and Nitrosomonas (Aly et al., 2008; Balcázar et al., 2007a; Gatesoupe, 1994; Rengpipat et al., 2000; Venkat et al., 2004), which is most commonly caused by some species of Vibrio (Gómez-Gil et al., 2004; Mujeeb-Rahiman et al., 2010). Gram-positive bacteria such as Bacillus offer an alternative to antibiotic therapy for shrimp farming. These bacteria are commonly found in marine sediments and are therefore naturally ingested by shrimp (Moriarty, 1999). Vibrio and Pseudomonas are the most common genera associated with aquatic environments (Otta et al., 1999), and both have also been periodically recommended as potential probiotic bacteria for cultivating fish, mollusks, and crustaceans.

A common way to select probiotics is to perform in vitro antagonism test. However, in vitro activity assays cannot be used to predict a possible in vivo effect (Balcázar et al., 2006). Therefore, it is essential to know the origin of the strain to survive and colonize the gastrointestinal tract of the host (Vine et al., 2004). Some Bacillus sp. produce hemolysins, which could be a health risk to the host (Liu et al., 2009b). In this study, hemolytic activity was negative for the isolates selected; thus these isolates were not a health risk. The antagonism test showed that probiotic strains were able to inhibit pathogenic strains of *V. harveyi* (CAIM 1793), *V. parahaemolyticus* (CAIM 170), *V. campbelli* (CAIM 333), *V. alginolyticus* (CAIM 57), and *V. vulniﬁcus* (CAIM 157). Similar results were obtained by Balcázar et al. (2007a), where *B. subtilis* UTM 126 was able to inhibit *V. parahaemolyticus* PS-107. Nakayama et al. (2009) found that cell-free

### Table 2

Test for antagonism of potential probiotic isolates against pathogenic *Vibrio* strains.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram</th>
<th>Hemolytic activity (Erythrocytes, Hemocytes)</th>
<th>Inhibition zone (mm)</th>
<th><em>V. parahaemolyticus</em></th>
<th><em>V. harveyi</em></th>
<th><em>V. campbelli</em></th>
<th><em>V. vulniﬁcus</em></th>
<th><em>V. alginolyticus</em></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Erythrocytes</td>
<td>Hemocytes</td>
<td>CAIM 170</td>
<td>CAIM 1793</td>
<td>CAIM 333</td>
<td>CAIM 157</td>
<td>CAIM 57</td>
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<tr>
<td>YC5-2**</td>
<td>+</td>
<td>γ NR</td>
<td>17.5±0.7</td>
<td>11±1.8</td>
<td>5±1.4</td>
<td>18±1.4</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>YC2-a**</td>
<td>+</td>
<td>γ NR</td>
<td>13.5±1.0</td>
<td>12±3.0</td>
<td>9±1.4</td>
<td>6.5±0.2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>C2-2</td>
<td>+</td>
<td>γ NR</td>
<td>21.5±1.1</td>
<td>11.5±2.1</td>
<td>NR</td>
<td>NR</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>YC3-b</td>
<td>+</td>
<td>γ NR</td>
<td>13.5±2.1</td>
<td>11±2.1</td>
<td>NR</td>
<td>NR</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>YC1-a</td>
<td>+</td>
<td>α NR</td>
<td>4.5±0.7</td>
<td>16.5±2.1</td>
<td>8.85±0.5</td>
<td>9±0.5</td>
<td>21.9±1.6</td>
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<tr>
<td>YC3-c</td>
<td>+</td>
<td>α NR</td>
<td>4.5±1.4</td>
<td>17.5±0.7</td>
<td>10±1.4</td>
<td>9.1±0.1</td>
<td>18.7±1.1</td>
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</tr>
<tr>
<td>YC3-a</td>
<td>+</td>
<td>α NR</td>
<td>3.5±0.7</td>
<td>13.4±1.1</td>
<td>8±1.4</td>
<td>8.15±1.6</td>
<td>18.1±1.8</td>
<td>*</td>
</tr>
<tr>
<td>YC2-b</td>
<td>+</td>
<td>β NR</td>
<td>8.5±0.7</td>
<td>8.5±2.1</td>
<td>13±1</td>
<td>NR</td>
<td>4.6±0.8</td>
<td>*</td>
</tr>
<tr>
<td>YC3-d</td>
<td>+</td>
<td>β NR</td>
<td>8.7±0.3</td>
<td>9.5±0.7</td>
<td>11±1</td>
<td>NR</td>
<td>10.75±0.4</td>
<td>*</td>
</tr>
</tbody>
</table>

* = Bacteriostatic effect.
** = Inhibitory effect for the two-layer method (Dopazo et al., 1988).
γ = Growth, but not hemolysis.
NR = Negative to the test.

### Table 3

Growth of bacterial in mucus of shrimp *Litopenaeus vannamei*.

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>CFU mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (h)</td>
<td>24</td>
</tr>
<tr>
<td>YC3-b</td>
<td>1.87×10⁶</td>
</tr>
<tr>
<td>C2-2</td>
<td>4.14×10⁶</td>
</tr>
<tr>
<td>YC5-2</td>
<td>&gt;10×10⁶</td>
</tr>
<tr>
<td>YC2-a</td>
<td>18×10⁶</td>
</tr>
<tr>
<td>YC3-a</td>
<td>&gt;10×10⁶</td>
</tr>
<tr>
<td>YC3-c</td>
<td>&gt;10×10⁶</td>
</tr>
<tr>
<td>YC1-a</td>
<td>&gt;10×10⁶</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Testing of adhesion of bacterial isolates to shrimp mucus and mucin by the Dot blot method. (−) = negative control (Buffer Hepes-Hanks) Capacity: weak adhesion (+), moderate adhesion (++), strong adhesion (+++).
supernatant from *B. subtilis*, *B. licheniformis*, and *B. megaterium* inhibited growth of one *V. harveyi* strain for 24 h. Decamp et al. (2008) administered *B. subtilis* and *B. licheniformis* to larval *L. vannamei* and *Penaeus monodon* and this inhibited growth of *Vibrio* strains and increased the survival rate of the shrimp. Inhibitory effects of *Bacillus* are attributed to various causes: alterations of the pH in growth medium, use of essential nutrients, and production of volatile compounds (Chaurasia et al., 2005; Gullian et al., 2004; Yilmaz et al., 2006). *Bacillus* spp. also produce polypeptides (bacitracin, gramicidin S, polymyxin, and tyrothricin) that are active against a broad range of Gram positive and Gram negative bacteria, which also explains the inhibitory effect on pathogenic *Vibrio* (Drablos et al., 1999; Morikawa et al., 1992; Perez et al., 1993).

During characterization of potential probiotics, we scrutinized their ability to adhere and colonize the intestine of shrimp. Adhesive bacterial strains proliferated, which was visible by epifluorescence microscopy, confirming that the isolates were capable of adhering to intestinal mucus and gastric mucin of *L. vannamei*. The number of viable cells increased in the mucus at 24 h and declined at 48 h, showing abundant free spores (Fig. 3). Similar studies reported that strains of *Bacillus* spp. are able to grow in water and colonize the digestive tract of shrimp. This ability is related to competitive exclusion.

**Fig. 3.** Bacterial strains stained with acridine orange growing in the intestinal mucus of *Litopenaeus vannamei* at 24 and 48 h of culture. (→) free spores in mucus. Growth observed by fluorescence microscopy at 100× magnification.
between introduced Bacillus strains with other bacteria (pathogens) for nutrients, space, or production of bacteriocins ( Moriarty, 1998; Rengpipat et al., 1998; Verschuere et al., 2000). Duc et al. (2004) reported that Bacillus spores can germinate in the human and animal gastrointestinal tract, but the mechanisms by which this is done is unclear. Currently, it is unknown whether Bacillus cells, spores, or both are responsible for competitive exclusion and probiotic effects that are attributed to several Bacillus species that allow them to transit successfully between animal gut and soil ( Patterson and Burkholder, 2003; Sanders et al., 2003). The adhesion and colonization of beneficial microbiota on intestinal mucus is essential to improve physiological response of the host (Alander et al., 1999). Therefore, it is important to design further studies focusing on appropriate frequency of administration (daily, every 3 days, weekly) of probiotics, and single or mixed doses to improve survival, growth, immune response, and disease resistance of cultured organisms.

Highest larval survival occurred with single-strain treatments, but the highest rate of larval development was obtained with the Mix-2 treatment. The difference in survival between the two bioassays can be explained by genetic variability of the shrimp broodstock and different management in the laboratory. Water quality in culture tanks is important to improve larval survival. Gram-positive Bacillus spp. are able to transform organic matter to CO₂ and it is used to produce bacterial biomass (Dalmin et al., 2001). In this study, the probiotic strains were inoculated directly to culture tanks to improve larval survival, but they also may be used to remove organic matter from culture systems.

The low survival of the control shrimp (5%) in the second trial reinforced the view that probiotics are highly effective for increasing survival of larvae. Srinivasas et al. (2010) showed that traditional practices (large exchange of water, application of disinfectants and antimicrobials, or both) are required to successfully complete the larval cycle; hence, the low survival rate in our control group in our bioassay was expected.

Because disease outbreaks cause major economic losses, a wide range of chemotherapeutics has been used to reduce bacterial disease. Oxytetracycline (OTX) is the most commonly used antibiotic in hatcheries in northwestern Mexico to prevent vibriosis by V. angiolyticus or V. harveyi (Uno et al., 2010). In our study, OTX-treated larvae had higher survival and a better index of development, compared to the control. However, OTX was not as efficacious as some probiotics.

The onset of exogenous feeding by larvae of penaeid shrimp is a critical phase in survival, growth, and development because the larval gut is exposed to microbes at the transition from nauplii 5 to zoea I (Jones et al., 1997). In our study, Bacillus tequilensis (strain YC5-2), B. endophyticus (strains C2-2 and YC3-b), and B. subtilis (strain YC2-a) significantly increased survival of larvae.

Using probiotics, modification of bacterial communities in tank water improves cultivation of larval crustaceans (Balcazar et al., 2007b; Garriques and Arenal, 1995; Gómez et al., 2008; Guo et al., 2009; Nogami and Maeda, 1992) and bivalves Douillet and Langdon (1993, 1994; Riquelme et al., 1996, 1997, 2001). Our study advances previous work demonstrating that probiotics maintain a balanced and natural bacterial community that improves production of shrimp larvae, which is also reflected in the rate of development, as demonstrated in our two bioassays with Bacillus spp.

5. Conclusions

In summary, selected strains of Bacillus are good candidates for probiotic application. This was the first time that Bacillus endophyticus and B. tequilensis are reported as potential probiotic strains. The isolates we tested were antagonistic to pathogenic strains of Vibrio and were not harmful to the larvae. Their ability to adhere and grow in intestinal mucosa is an important factor in colonizing or at least remaining for short time periods in the gut of shrimp. More rapid development also occurred when the larvae were treated with mixtures of Bacillus strains. Treatment Mix-2 increased survival and larval development, compared to the control group. Similar results were

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Table 4

Rate of development of Litopenaeus vannamei larvae inoculated with bacterial isolates (C2-2, YC2-a, YC3-2, and YC3-b), commercial probiotics (Alibio™ and Epicin™), and oxytetracycline (OTX), control (larvae without bacteria), Mix-1 (isolates C2-2 and YC3-b, 1:1), Mix-2 (isolates C2-2, YC3-b, and YC3-2 at 1:1:1 proportion).

<table>
<thead>
<tr>
<th>Assay</th>
<th>YC2-a</th>
<th>C2-2</th>
<th>YC3-b</th>
<th>YC3-2</th>
<th>Epicin™</th>
<th>control</th>
<th>Mix-1</th>
<th>Mix-2</th>
<th>OTX</th>
<th>Alibio™</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.38 ± 0.2a 6.54 ± 0.1bc</td>
<td>6.56 ± 0.2bc</td>
<td>6.45 ± 0.2bc</td>
<td>6.73 ± 0.1bc</td>
<td>6.00 ± 0.0a 6.68 ± 0.3a</td>
<td>5.70 ± 0.4a</td>
<td>7.00 ± 0.1a</td>
<td>6.53 ± 0.1bc</td>
<td>6.55 ± 0.0bc</td>
<td>6.35 ± 0.2bc</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.88 ± 0.3a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values with same letter are not significantly different (P > 0.05).
found by Guo et al. (2009), where B. fusiformis increased survival and accelerated metamorphosis of P. monodon and L. vannamei larvae. This study demonstrated that management that combines properly selected Bacillus isolates are a good option in larviculture to improve health, rate of development, and rate of survival of whiteleg shrimp.

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