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Research Article

Effect of bacterial probiotics bio-encapsulated into *Artemia franciscana* on weight and length of the shortfin silverside (*Chirostoma humboldtianum*), and PCR-DGGE characterization of its intestinal bacterial community

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ABSTRACT. The shortfin silverside (*Chirostoma humboldtianum*) is a native fish of central Mexico with high value for artisanal fisheries. So far, attempts aimed to establish intensive culturing have failed. In this study, we evaluated the effect of probiotic strains; *Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus johnsonii* C4, and *Bacillus* sp. B2 bio-encapsulated into *Artemia franciscana* on *Chirostoma humboldtianum* weight and length. Their influence on the fish intestinal bacterial communities was also assessed. The final weight and final length of the fishes fed with bio-encapsulated *Bifidobacterium animalis* BB-12, and *L. johnsonii* C4 were statistically different and higher than the control group. According to PCR-DGGE fingerprints of 16S rRNA gene, the intestinal content bacterial community associated with the shortfin silverside seems to be molded in early larval stages and only slight changes could be induced by the use of bio-encapsulated bacterial. An increase in fish survival rate and an improvement in weight and length were detected using *L. johnsonii* C4 bio-encapsulated into *A. franciscana,* in spite of its small impact on the structure of the bacterial community associated with the intestinal content of shortfin silverside. The use of *L. johnsonii* C4 bio-encapsulated into *A. franciscana* could be an excellent option to improve the yield during intensive culturing of the shortfin silverside.

Keywords: *Chirostoma humboldtianum*, *Artemia franciscana*, bacterial probiotics, bio-encapsulated, weight, length, bacterial communities.

INTRODUCTION

The shortfin silverside *Chirostoma humboldtianum* (Valenciennes, 1835), commonly known in Mexico as "pescado blanco", is a fish species endemic to central Mexico, which mainly lives in lakes and freshwater reservoirs. Since prehispanic times, this *Atherinopsidae* fish has been the basis for most of the artisanal fisheries from different Mexican ethnic groups. Besides its high nutritional qualities, its importance in Mexican artisanal fisheries is also due to its cultural, economic, and ecological significance (Rojas-Carrillo & Sasso-Yada, 2005; Martínez-Palacios *et al*., 2006). However, pollution, habitat reduction, the introduction of alien species, overexploitation and non-selective fishing have decreased shortfin silverside populations (Rojas-Carrillo & Sasso-Yada, 2005), affecting its distribution severely across the Valley of Mexico, Jalisco and Nayarit States. Particularly in Nayarit State and the Valley of Mexico, this species has been exterminated from lakes and channels (Paulo-Maya *et al*., 2000; Blancas-Arroyo *et al*., 2004; Bojórquez & Arana, 2014).

Production of *Chirostoma* spp. in the early 1980s reached 7,980 ton, while in 2003 production decreased dramatically to 812 ton. In 2010, the shortfin silverside production was estimated at 3,381 ton and seemed to be recovering, but there is still a lack of strategies for rea-

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ching a good level of aquaculture production (SAGARPA, 2010).

Initial attempts to culture members of genus *Chirostoma* have reported poor results because of high mortality in embryos and yolk-sac larvae, and the inability of early larval stages to digest artificial food (Figueroa-Lucero *et al*., 1999; Hernández-Rubio *et al*., 2006). The immature digestive system in early larval stages seems to be related to low digestive enzyme activities in the fish (Dabrowski & Glogowski, 1977; Lauff & Hofer, 1984; Pedersen *et al*., 1987; Gawlicka *et al*., 2000). To solve this issue, larval and juvenile fish could benefit from exogenous enzymes provided by live food to activate their gut zymogens (Dabrowski & Glogowski, 1977). The use of live food is essential to raise *Chirostoma* fish; larvae in the first exogenous feeding stage need rotifers like *Brachionus rubens, B. plicatilis* and *B. calyciflorus*, as its main food. After around 15 days post-hatching, rotifers are gradually replaced by *Artemia* spp. nauplii and finally, 30 days after hatching, larvae foodstuffs may be progressively replaced with artificial food (Figueroa-Lucero *et al*., 2004; Martínez-Palacios *et al*., 2006). However, despite the initial use of live food in *Chirostoma* spp. culture systems, studies are reporting low larval survival rates after shifting to an artificial diet (Figueroa-Lucero *et al*., 2003; Martínez-Palacios *et al*., 2006).

A possible alternative to improve survival rates in *Chirostoma* spp. culture systems could be the use of probiotics in well-defined preparations of viable microorganisms, with concentrations high enough to modify the gut microbiota composition and exert beneficial health effects to the host (Schrezenmeir & de Vrese, 2001). Probiotics have been successfully used in several fish species increasing survival rates and yield (Wang & Xu, 2006; Aly *et al*., 2008; Bagheri *et al*., 2008; Vendrell *et al*., 2008). Probiotics enhance the bioavailability of proteins, nutrients, and digestive enzymes (Gatesoupe, 2008; Sen *et al*., 2012), stimulate the immune system and modulate intestinal microbiota (Verschuere *et al*., 2000; Burr *et al*., 2005). Bacteria from the genera *Pseudomonas, Bacillus, Bifidobacterium, Streptococcus, Enterococcus, Lactobacillus* and the yeast *Saccharomyces* are the most widely studied probiotics regarding their use in aquaculture production (Gatesoupe, 1994; Balcazar *et al*., 2007b, 2008; Denev *et al*., 2009; Martínez-Cruz *et al*., 2012).

For example, *Bacillus* spp. and *Lactobacillus* spp. had been successfully used in the rainbow trout *Oncorhynchus mykiss* (Walbaum), the common carp *Cyprinus carpio* (Linnaeus) and the Nile tilapia *Oreochromis niloticus* (Linnaeus) to increase pathogen resistance and/or fish fitness (Nikoskelainen *et al*., 2001; Raida *et al*., 2003; Pirarat *et al*., 2006; Wang & Xu, 2006; Aly *et al*., 2008; Bagheri *et al*., 2008). Likewise, in the brown trout *Salmo trutta* (Linnaeus) gut microbiota was modified and the humoral immune response was stimulated using commercial food supplemented with *Lactococcus, Lactobacillus* and *Leuconostoc* strains (Balcazar *et al*., 2006b, 2007a). Moreover, *Bifidobacterium bifidum* plus *Lactobacillus acidophilus*, *Lactobacillus casei* and *Enterococcus faecium* increased growth performance and survival rates of Persian sturgeon *Acipenser persicus* (Borodin) juveniles when probiotic bacteria were added to the artificial commercial diet.

Rotifers and brine shrimps (*Artemia* spp.) feed with probiotic bacterial cells may act as carriers when they are used as live food for fish. After being ingested, they release probiotics inside the fish alimentary canal (Gatesoupe, 1994; Dhont & Sorgeloos, 2002). Due to its filtering capacity and to the ability to ingest floating particles, *Artemia franciscana* (brine shrimp) is the main live food used in larviculture for probiotics bio-encapsulation (Sorgeloos *et al*., 2001). Fish diseases and nutritional deficiencies have been successfully treated by bio-encapsulation in brine shrimp of yeasts, oils, liposomes, bacteria, emulsions, antibiotic, vitamins and amino acids (Gomez-Gil *et al*., 2000; Tonheim *et al*., 2000; Gelabert-Fernández, 2001). Considering that the direct administration of probiotic cells into culture water results in a fast and high mortality of bacterial cells and high rates of microbial contamination, the probiotic bio-encapsulation in brine shrimp is an excellent alternative to avoid such difficulties (Gatesoupe, 2008).

Currently, there is a global tendency to reduce the use of antibiotics and other chemicals that cause adverse effects to the host and the environment. Modern aquaculture practices have focused on the use of probiotics as an alternative therapy to reduce mortality and enhance aquatic species production in an environmentally friendly way (Romero *et al*., 2012). Hence, this study was focused on the effect of administrating the probiotic bacterial strains; *Bifidobacterium animalis* subsp. *lactis* strain BB-12, *Lactobacillus johnsonii* C4 and *Bacillus* sp. B2, bio-encapsulated into *Artemia franciscana,* on the growth and gut bacterial community composition of the shortfin silverside (*Chirostoma humboldtianum*).

MATERIALS AND METHODS

Probiotic bacteria

Probiotic strains used in the study were: *Bifidobacterium animalis* subsp. *lactis* strain BB-12

(Christian Hansen), *Lactobacillus johnsonii* C4 (Roy *et al*., 2000) and *Bacillus* sp. strain B2 (Monroy-Dosta *et al*., 2010). *B. animalis* subsp. *lactis* strain BB-12 and *L. johnsonii* C4 were grown for 12 h. in TPYG broth (Trypticase-Peptone-Yeast extract-Glucose), pH 7.0 under anaerobic conditions. *Bacillus* sp. B2 was grown for six h in Trypticasein Soy Broth (TSB Bioxon, Becton Dickinson, Mexico) under aerobic conditions. All cultures were incubated at 28°C with shaking at 200 rpm. Cell counts were determined by a pour-plate method in MRS agar (Difco Lactobacilli MRS Agar, Becton Dickinson), enriched with 0.05 g L^{-1} L-cysteine, 0.02 g L^{-1} $NaCO_3$, and 0.01 g L^{-1} $CaCl_2$. *B. animalis* subsp. *lactis* strain BB-12 and *L. johnsonii* C4 cultures and counts were carried out in an anaerobic chamber (Anaerobic System, Model 1025, Forma Scientific, Marietta, OH, USA) containing an atmosphere of 5% H_2, 10% CO_2, and 85% N_2; while, *Bacillus* sp. B2 was incubated under aerobic conditions. All counting-cultures were done in triplicate and incubated for 24 h at 28°C. Probiotic cell suspensions were prepared monthly. Sterile glycerol solution (50% v/v) was added to cell suspensions until it reaches a final glycerol concentration of 20% v/v. Glycerol-cell stock suspensions were stored at -20°C and their viability were verified twice a week to ensure a final concentration of 10^8 CFU in the suspensions used to feed *A. franciscana* metanauplii.

Artemia franciscana culture and probiotic bio-encapsulation

Bacteria-free *A. franciscana* cysts (Great Salt Lake strain, INVE Aquaculture *Artemia* Systems, Baasrode, Belgium) were incubated in acrylic cones containing sterile water at 30 g L^{-1} salinity, with continuous illumination (2.000 lux) and permanent dissolved oxygen concentration (4.5 ± 0.6 mg L^{-1}), for 24 h at 28 ± 1ºC. Positively phototactic nauplii were harvested and transferred to new vessels and fed with the microalgae *Tetraselmis suecica* until metanauplii phase was reached after approximately 48 h (Castro *et al*., 2005). Bio-encapsulation of probiotic strains; *B. animalis* subsp. *lactis* BB12, *L. johnsonii* C4, and *Bacillus* sp. B2 in *A. franciscana* metanauplii was carried out following a method described by Patra & Mohamed (2003). In brief, metanauplii were starved for 15 h by transferring them into vessels with a sterilized saline solution (30 g L^{-1}) at a density of 240 metanauplii L^{-1}. Then, each probiotic strain was added to achieve a final concentration of 10^8 CFU mL^{-1}. Metanauplii and bacterial cells were incubated with gentle agitation for 40 min. Finally, metanauplii alimentary canals were observed with a stereoscopic microscope to verify their saturation with bacterial cells (Castro *et al*., 2005).

Production of *Chirostoma humboldtianum* larvae

In order to obtain larvae for the feeding trial, wild adult fishes (total length 12-18 cm) were captured in Villa Victoria Dam, State of Mexico. Net fishing was carried out with a 500×1 m hammock type net, with 6 mm mesh light. A total of 113 fishes of required sizes were captured after three net trawls. Selected fishes were placed in plastic bags with filtered water from the dam. To reduce post-capture and transport stresses, NaCl (10 g L^{-1}), and 0.05% V/V Pentabiocare Colloidal solution (Biomaa®, Mexico) were added to the water. Bags headspace was filled with oxygen and then sealed. Ambient temperature was maintained at 18°C through the four h trip to the facilities at the Center for Biological and Aquaculture Research, in our Institution. The specimens were confined and acclimatized in circular ponds (3 m diameter×0.80 m depth) with air supply. Food consisted of small forage fishes, amphipods, and cladocerans, that were available *ad libitum*. Twice a week 50% water changes were made to maintain water quality. Although appropriate conditions were maintained for captive maintenance, adult survival after 45 days was only 20% (22 ind). To induce reproduction, changes were made in the water level of the ponds during fifteen days, in addition to the placement of vegetation as substrates for oviposition. After larvae hatching, the initial feeding for their maintenance consisted of a diet composed of rotifers (*Brachionus plicatilis* and *B. rubens*), nematodes (*Panagrellus redivivus*) and *Artemia franciscana* nauplii for three weeks, after that period fishes were ready for consumption of *Artemia* methanauplii for the probiotics trial. Finally, at this stage 75 larvae were obtained from 185 eggs of *C. humboldtianum*, this was the maximum number of same-size larvae that we could obtain.

Live-prey feeding assay

The 21 days old shortfin silverside fingerlings measuring 1.36 ± 0.014 cm total length, and weighing 0.045 ± 0.003 g (mean ± SE) were randomly distributed into four containers (12 L each), with 18 individuals each one, accounting for a total of 72 fingerlings. Four food-treatments were set up as follows: 1) *A. franciscana* metanauplii bio-encapsulating *B. animalis lactis*, 2) *A. franciscana* metanauplii bio-encapsulating *L. johnsonii* C4, 3) *A. franciscana* metanauplii bio-encapsulating *Bacillus* sp. B2, and 4) Control group fed with probiotic-free *A. franciscana* metanauplii. Each fish was considered an experimental unit. Shortfin silversides were fed twice a day with the corresponding probiotic enriched or probiotic-free *A. franciscana* (720 metanauplii/container/day), plus a balanced formula for trout containing 45% protein (El Pedregal ®, Silver

Cup Mexico, fine crumb < 0.6 mm). During the first month, each *C. humboldtianum* fingerling was fed with an average of 40 metanauplii/individual/day. This initial ratio was increased with 10 preys/individual/day each month. Also, balanced food was provided at a feeding rate of 7% of total biomass per day, this was calculated by weekly weighing half of the fishes in each pond. The daily food amount was adjusted to the number of surviving fishes in each tank. Feces and uneaten food were removed after every meal and water level was restored.

The assay was carried out at 22 ± 1°C in a room-controlled temperature, with 12 h photoperiod, under the following water parameters; pH 8.0 ± 0.1, dissolved oxygen 4.5 ± 0.5 mg L^{-1}, nitrates 0.50 ± 0.3 mg L^{-1} and ammonium 0.032 ± 0.01 mg L^{-1}, that were monitored weekly during the 30 weeks of the experiment. Aeration of the containers was provided with an air pump to keep the dissolved oxygen at the values mentioned above. Fish weight and total length (from the tip of the snout to the end of the caudal fin) were recorded at the beginning, weekly (weight only) and the end of the experiment when fishes were sacrificed. To avoid fish stress, fishes were anesthetized with 13 µL L^{-1} of clove oil during 30 min. Each month, the fingerlings were weighted using an Ohaus Adventurer™ AV114 Pro analytical balance, and length was assessed with a Control Company Traceable® digital caliper with ± 0.03 mm accuracy. As soon as measurements were done, the fingerlings were placed in a container with fresh water during 5 min. Then, the individuals were relocated in their respective container (Vázquez *et al*., 2013). The fingerlings were euthanized by an overdose of clove oil (1 mL L^{-1}) during 5 min. Handling and euthanizing procedures were according to the guidelines of The Academic and Ethical Committee of El Hombre y Su Ambiente Department, at Universidad Autónoma Metropolitana, Unidad Xochimilco. Finally, fish survival rates (S) in each treatment were recorded.

Statistical analysis

Since the length and weight values did not meet the normality assumption, data values were normalized using a two-step transformation (Templeton, 2011). Weight-normalized values were compared using *t*-test. Whereas, length normalized values were compared by a Welch's *t*-test, as Levene's test indicated unequal variance. The level of significance was set at $\alpha = 0.05$ in all tests (Hogg & Tanis, 2005).

Molecular analysis of bacterial communities

At the end of the experiment, fingerlings were starved for 24 h, then euthanized and eviscerated using the sterile material, under aseptic conditions to obtain the gastrointestinal tract. The hindguts were squeezed and their contents transferred into 1.5 mL microtubes. The intestinal contents of all survivor fingerlings of each treatment were placed and homogenized in the same 1.5 mL microtube. Metagenomic DNA from intestinal contents and genomic DNA from probiotic bacteria were extracted as described previously (Morales-Jiménez *et al*., 2012).

Bacterial communities from intestinal contents of fishes were compared using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis. Metagenomic DNA obtained from the intestinal contents of fingerlings from control and probiotic treatments were used as a target for PCR amplification of V3 and V6-V8 regions of 16S rRNA gene. DGGE primers, PCR conditions, and DGGE general methodology were performed following directions previously described (Morales-Jiménez *et al*., 2012). PCR-DGGE profiles displayed by both 16S rRNA regions and the different experimental groups were grouped using Dice similarity coefficient and UPGMA hierarchical clustering method, the image analysis was made with the software Syngene (Synoptics Ltd., UK). Selected DGGE bands were excised from gels, reamplified with the same primers without GC-clamp and then sequenced as described previously (Ramírez-Saad *et al*., 2000). Maximum likelihood phylogenetic analysis of sequences obtained from reamplified DGGE bands was performed. Sequences generated in this study were deposited in GenBank under accession numbers KJ004748-KJ004759 or as supplementary material for the V3 region of 16S rRNA gene, as sequences were too short to be submitted to GenBank.

RESULTS

Fish fitness

After 30 weeks of the experiment, the shortfin silverside development measured as normalized final weight showed that shortfin silversides fed with *L. johnsonii* C4 and *B. animalis* subsp. *lactis* strain BB-12 bio-encapsulated into *A. franciscana* (Table 1, Fig. 1) were heavier than fingerlings in control group. Normalized final length of fingerlings fed with *L. johnsonii* C4 and *B. animalis lactis* BB-12 was statistically larger than the normalized final length of fingerlings in control group (Table 1, Fig. 1).

Meanwhile, the normalized final weights and lengths of fingerlings treated with *Bacillus* sp. B2 was not statistically different from the normalized final weights and lengths of fingerlings of the control group. Finally, the survival rate of fishes fed with *L. johnsonii*

Table 1. Fitness parameters of short fin silverside fingerlings fed with *A. franciscana* bio-encapsulating with probiotic bacteria. Values are means ± standard deviation. *Indicates a statistically significant difference with control group ($P < 0.05$) using a *t*-test or Welch's *t*-test. For details, see Fig. 1.

Parameters	Probiotics			
	Bifidobacterium animalis subsp. *lactis* strain BB-12	*Lactobacillus johnsonii* C4	*Bacillus* sp. B2	Control without probiotic
Initial weight (g)	0.047 ± 0.005	0.046 ± 0.005	0.041 ± 0.005	0.047 ± 0.005
Normalized final weight (g)	3.10 ± 0.39*	4.22 ± 0.57*	2.06 ± 0.67	2.45 ± 0.43
Initial length (cm)	1.36 ± 0.03	1.37 ± 0.03	1.34 ± 0.03	1.38 ± 0.03
Normalized final length (cm)	7.71 ± 0.25*	8.52 ± 0.40*	6.95 ± 0.59	7.18 ± 0.15
Final *n*	9	14	9	7

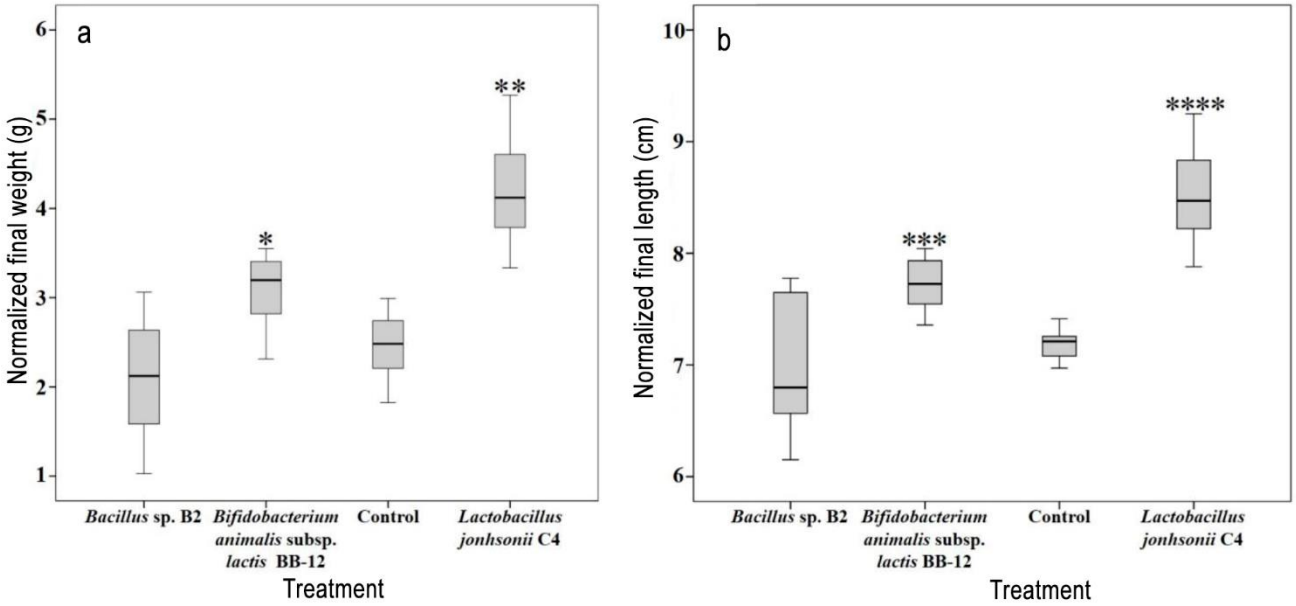

Figure 1. a) The normalized final weight of *Chirostoma humboldtianum* fingerlings fed with *Artemia franciscana* enriched with different probiotic bacteria and control group without bacteria, b) normalized final length of *Chirostoma humboldtianum* fingerlings fed with *Artemia franciscana* enriched with different probiotic bacteria control group without bacteria. *indicates that fingerlings fed with *B. animalis* subsp. *lactis* strain BB-12 bio-encapsuled in *A. franciscana* (M = 3.1079, SD = 0.3954) were heavier than fingerlings in control group (M = 2.4566, SD = 0.4311), t (14) = 3.144, P = 0.007, d = 1.57. **Indicates that fingerlings fed with *Lactobacillus johnsonni* C4 bio-encapsuled in *A. franciscana* (M = 4.2231, SD = 0.5783) were heavier than fingerlings in control group (M = 2.4566, SD = 0.4311), t(19) = 7.116, $P < 0.001$, d = 3.46. ***Indicates that fingerlings fed with *B. animalis* subsp. *lactis* strain BB-12bio-encapsuled in *A. franciscana* (M = 7.7181, SD = 0.2470) were bigger than fingerlings in control group (M = 7.1810, SD = 00.1509), t(14) = 5.012, $P < 0.001$ d = 2.62. ****Indicates that fingerlings fed with *Lactobacillus johnsonni* C4 bio-encapsuled in *A. franciscana* (M: 8.5297, SD: 0.1509) were bigger than fingerlings in control group (M: 7.1810, SD: 0.2470), t: (18.2) = 11.043, $P < 0.001$ d: 4.42. Levene's test indicated unequal variance (F = 6.087, P = 0.023). M: means mean, SD: means standard deviation, t: means *t*-test and d: effect size.

C4 bio-encapsulated into *A. franciscana* was twice than the survival rate of the control group. The control group had a survival rate of 38.8% meanwhile the group treated with *L. johnsonii* C4 showed a survival rate of 77.7%. Fishes fed with *B. animalis* subsp. *lactis* strain BB-12 and *Bacillus* sp B2 exhibited a survival rate of 50%, an increase of 11.2% compared with the survival rate of the control group.

Analysis of microbial communities

PCR-DGGE profiles of the V3 region of 16S rRNA gene showed from 18 to 20 bands per sample (Fig. 1),

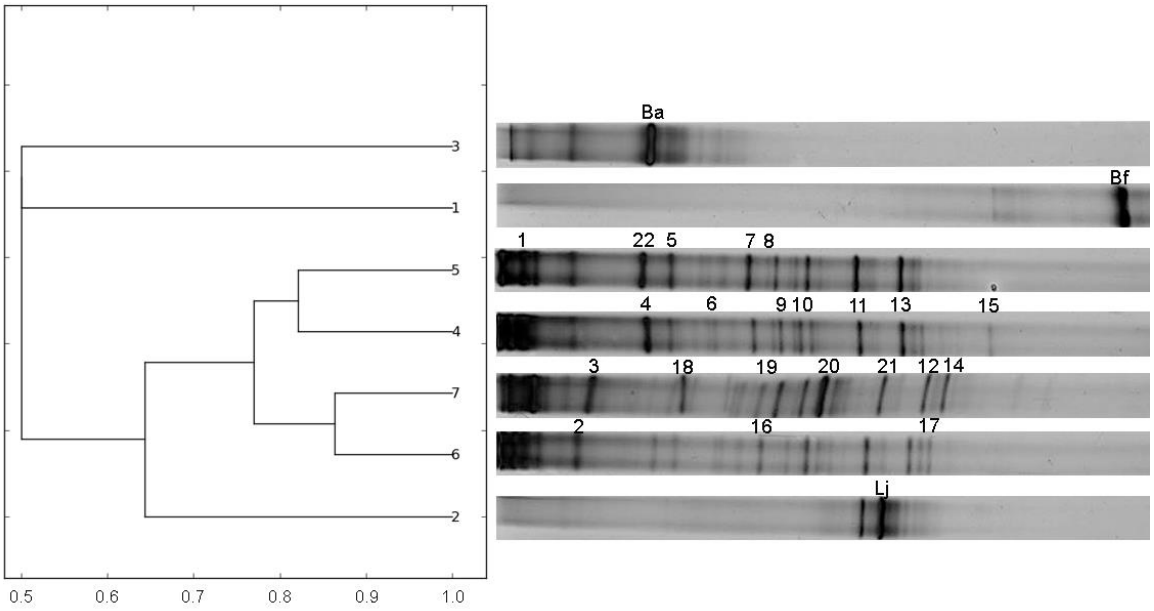

Figure 2. PCR-DGGE-profiles and dendrogram constructed from amplicons from the V3 region of 16S rRNA gene. PCR products were obtained from genomic DNA of the following probiotic bacteria; *B. animalis lactis* (lane 1); *L. johnsonii* strain C4 (lane 2); *Bacillus* sp strain B2 (lane 3). Lanes 4-7 showed similar amplicons but obtained from metagenomic DNA from fecal samples of Shortfin Silverside fed as follows: lane 4 -control group (*A. franciscana* without probiotic bacteria); lane 5 -*A. franciscana* bio-encapsulating *B. animalis* subsp. *lactis* strain BB-12; lane 6 -*A. franciscana* bio-encapsulating *L. johnsonii* C4; lane 7 -*A. franciscana* bio-encapsulating *Bacillus* sp B2. Black numbers indicate DGGE bands that were excised and sequenced, microbial affiliations of those DGGE bands are summarized in Table 2. The bar numbers indicate the Euclidean distances.

where as the profiles of regions V6-V8 of 16S rRNA gene showed from 9 to 17 bands per sample (Fig. 2). Despite the differences in a number of bands, dendrograms from both regions showed similar clustering patterns, with *Bacillus* and *Lactobacillus* treatments forming the most coherent group (*ca*. 0.8 S_{AB}). These patterns were joining to *Bifidobacterium* at 0.6 S_{AB}, being the control treatment the less similar to the former three with a S_{AB} value <0.5.

PCR-DGGE profiles of V3 region of 16S rRNA gene showed no significant changes in fish intestinal content microbiota among all treatments (Fig. 2). Twenty-two DGGE bands from these profiles were sequenced; 63.6% of them belonged to γ-*Proteobacteria*, 31.8% to non-cultured bacteria, and 4.5% to unidentified bacterial isolates.

The dominant genus was *Vibrio* (50%), followed by non-culturable bacteria (31.8%). Members of *Alteromonas* (4.54%), *Idiomarina* (4.54%), and *Serratia* (4.54%) were also detected (Table 2).

PCR-DGGE fingerprints of V6-V8 16S rRNA gene of fish fecal bacterial communities neither displayed striking differences among experimental groups (Fig. 3). A total of 12 bands were excised, reamplified and sequenced. Phylogenetic affiliations from sequences of these bands are shown in Figure 4. Taxonomic distribution of identified bacteria in fish supplemented with probiotics is summarized in Table 3. In brief, the intestinal content bacteria belong to the phyla *Proteobacteria* (58.4%) and *Firmicutes* (31.6 %). The identified genera were: *Allivibrio, Vibrio, Exiguobacterium,* and *Fusibacter*, which were present in all treatments. Members of genus *Rheinheimera* were only detected in fishes fed with *A. franciscana* enriched with *B. animalis* BB-12 or *Bacillus* sp. B2, whereas *Aeromonas* was exclusively recorded in feces from fish supplemented with *Bacillus* sp. B2.

DISCUSSION

Statistically significant increases in final weight and final length were detected when *B. animalis* subsp. *lactis* strain BB-12 or *L. johnsonii* C4 was added to the fish diet. Similar results have been reported in turbot fish *Psetta maxima* (Linnaeus) when *A. franciscana* enriched with two commercial formulations of probiotics containing *Lactobacillus* and *Enterococcus,* or *Lactobacillus* and *Pediococcus* were used as food (Dagá *et al*., 2013). Moreover, in the freshwater angelfish total body length was enhanced when fish

Table 2. Bacterial taxa affiliations of V3 16S rRNA region DGGE bands of fecal microbiota from the Shortfin silverside fed with *A. franciscana* enriched with bacterial probiotics. [+]Presence of DGGE band in the respective treatment. [a]*B. animalis* subsp. *lactis* strain BB-12, [b]*L. johnsonii* C4, [c]*Bacillus* sp. B2, [d]Control without probiotic.

DGGE Band	Phylogenetic relationships		Probiotics			
	Class	% Identity to GenBank sequence (accession number)	Bal[a]	C4[b]	B2[c]	C[d]
1	Undefined	98% Uncultured bacterium clone (KC259972)	+	+	+	+
2	γ-Proteobacteria	99% Uncultured bacterium clone (JQ579769)		+	+	
3	γ-Proteobacteria	100% *Vibrio vulnificus* (JQ307114)	+	+	+	+
4	γ-Proteobacteria	100% *Vibrio* sp. (DQ642844)			+	+
5	Undefined	100% Uncultured bacterium clone (HM018046)	+	+		+
6	γ-Proteobacteria	100% *Vibrio harveyi* (JF520422)		+		+
7	Undefined	100% Uncultured bacterium clone (JX940421)	+			
8	Undefined	99% Environmental bacterium clone TSA241-8 (HG792166)	+			+
9	γ-Proteobacteria	100% *Vibrio harveyi* (JF520418)	+		+	+
10	γ-Proteobacteria	100% *Vibrio harveyi* (JF520418)	+		+	+
11	γ-Proteobacteria	100% *Idiomarina loihiensis* (KF688213)	+	+	+	+
12	γ-Proteobacteria	99% *Serratia marcescens* (KC435001)	+	+	+	+
13	Undefined	100% Uncultured bacterium clone (KF601966)	+			+
14	Undefined	100% Uncultured bacterium clone (JX940421)			+	+
15	γ-Proteobacteria	99% *Vibrio harveyi* (JF520422)				+
16	γ-Proteobacteria	99% *Vibrio harveyi* (JF520422)		+		
17	γ-Proteobacteria	99% *Vibrio harveyi* (JF520422)		+		
18	Undefined	100% Uncultured bacterium clone (JX940421)				
19	γ-Proteobacteria	99% to *Vibrio* sp. (KC534342)			+	
20	γ-Proteobacteria	99% *Vibrio harveyi* (JF520422)			+	
21	γ-Proteobacteria	100% *Vibrio* sp. (KC534342)			+	
22	γ-Proteobacteria	99% *Alteromonas* sp. (KF010924)	+			

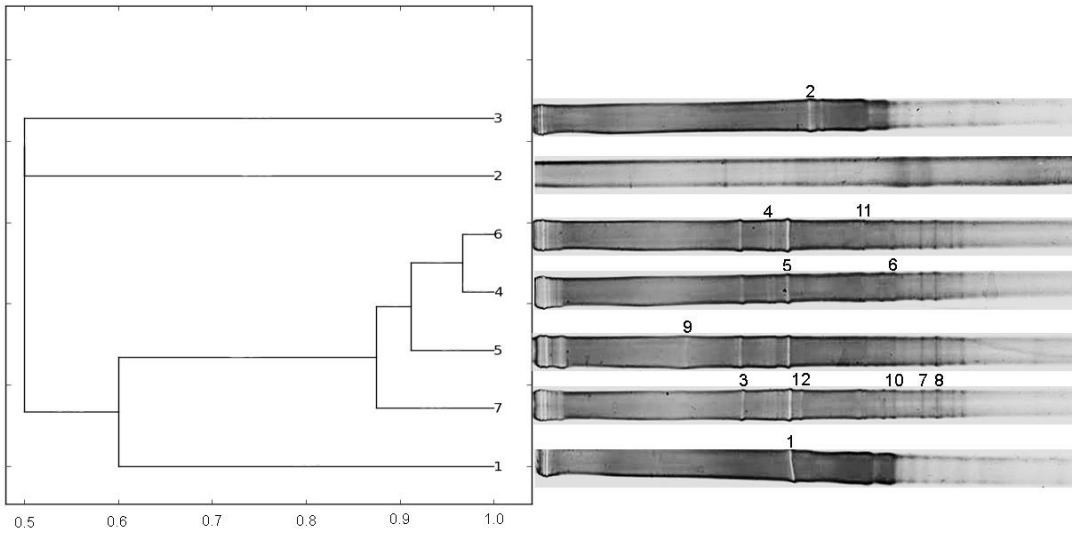

Figure 3. PCR-DGGE-profiles and dendrogram constructed from amplicons from the V6-V8 regions of the 16S rRNA gene. PCR products on lanes 1-3 were obtained from genomic DNA of the following bacterial probiotic strains: *Bacillus* sp B2 (lane 1), *B. animalis lactis* (lane 2), *L. johnsonii* C4 (lane 3). Profiles on lanes 4-7 were obtained from metagenomic DNA from fecal samples of shortfin silverside fed as follows: lane 4 -control group, *A. franciscana* without probiotic bacteria; lane 5 -*A. franciscana* bio-encapsulating *B. animalis* subsp. *lactis* strain BB-12; lane 6 -*A. franciscana* bio-encapsulating *L. johnsonii* C4; lane 7 -*A. franciscana* bio-encapsulating *Bacillus* sp B2. Black numbers indicate DGGE bands that were excised and sequenced microbial affiliations of those DGGE bands are summarized in Table 3. The bar numbers indicate the Euclidean distance.

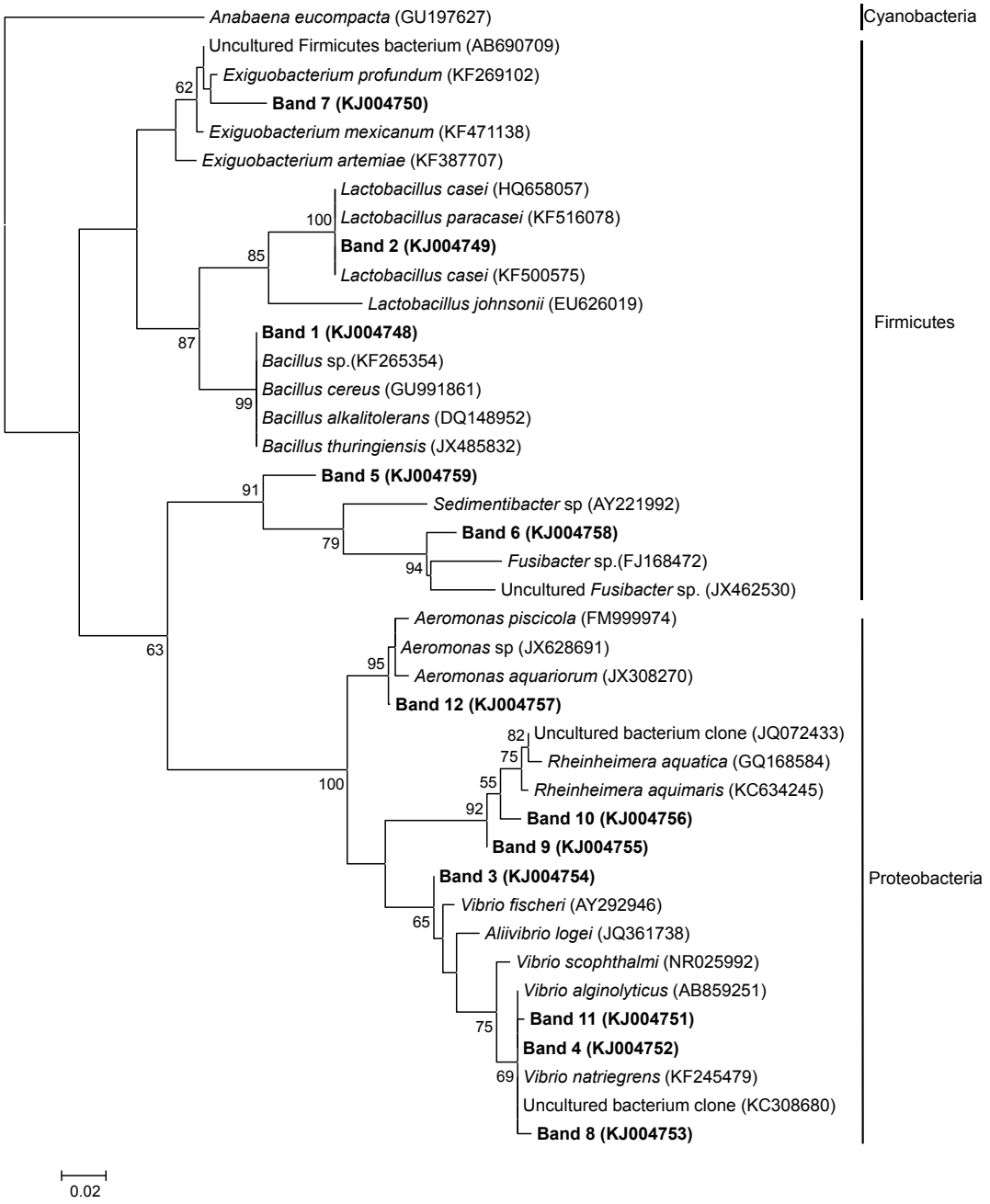

Figure 4. Maximum likelihood tree of PCR-DGGE bands from V6-V8 regions of 16S rRNA gene amplified from fecal samples of shortfin silverside. The partial 16S rRNA gene sequence of *Anabaena eucompacta* was used as an out-group. Scale bar indicates 10% estimated sequence divergence. Bootstrap support values are indicated for major nodes having values of ≥50%.

were fed with a mixture of *Bacillus* spp. bio-encapsulated into *Artemia urmiana* (Farahi *et al*., 2011). Similarly, bacterial probiotic addition without a live carrier has improved the growth of several fish species like the goldfish *Carassius auratus;* the green swordtail *Xiphophorus helleri;* the common carp, the Nile tilapia, and the rainbow trout (Ahilan *et al*., 2004; Wang & Xu, 2006; Abraham *et al*., 2008; Aly *et al*., 2008; Bagheri *et al*., 2008).

Dietary supplementation of probiotics increases protein availability due to the proteolytic activity of beneficial bacteria. Also, probiotics enhance fishes' fitness improving macronutrients digestibility by releasing essential nutrients (amino acids and vitamins)

Table 3. Bacterial taxa affiliations of V6-V8 16S rRNA region DGGE bands of fecal microbiota from Shortfin Silverside fed with *A. franciscana* enriched with bacterial probiotics. [+]Presence of DGGE band in the respective treatment. [a]*B. animalis* subsp. *lactis* strain BB-12, [b]*L. johnsonii* C4, [c]*Bacillus* sp. B2, [d]Control without probiotic.

DGGE Band	Phylogenetic relationships		Probiotics			
	Class	% Identity to GenBank sequence (accession number)	Bal[a]	C4[b]	B2[c]	C[d]
1	*Firmicutes*	99% to *Bacillus* sp. (KF265354)			+	
2	*Firmicutes*)	100% to *Lactobaciillus. casei* (HQ658057)	+			
3	*γ-Proteobacteria*	99% to *Aliivibrio fischeri* (AY292946)	+	+	+	+
4	*γ-Proteobacteria*	100% to Uncultured bacterium clone (KC308680)		+		+
5	*Firmicutes*	96% to *Fusibacter* sp. (FJ168472)	+	+	+	+
6	*Firmicutes*	96% to *Fusibacter* sp. (FJ168472)				+
7	*Firmicutes*	97% to Uncultured Firmicutes bacterium (AB690709)	+	+	+	+
8	*γ-Proteobacteria*	99% to Uncultured bacterium clone (KC308680)	+	+	+	+
9	*γ-Proteobacteria*	98% to Uncultured bacterium clone (JQ072433)	+			
10	*γ-Proteobacteria*	97% to *Rheinheimera aquimaris* (KC634245)	+			
11	*γ-Proteobacteria*	99% to Uncultured bacterium clone (KC308680)		+		
12	*γ-Proteobacteria*	99% to *Aeromonas* sp. (JX628691)			+	

and digestive enzymes and also improving micronutrients absorption (Balcazar *et al.*, 2006a). Particularly, *Bacillus* sp. strain B2 was able to improve the resilience to the colonization of *Aeromonas hydrophila* in *Pterophyllum scalare*, this strain also enhanced the growth performance and survival of the goldfish *Carassius auratus* (Monroy-Dosta *et al.*, 2010; Castro-Barrera *et al.*, 2011). Whereas, several strains of lactobacilli have been used to control gut bacterial infections in aquaculture, showing an extra effect on the growth rate of fishes (Ringø *et al.*, 2010). The application of *Bifidobacterium animalis* and *Lactobacillus johnsonii* in farm animals has been extensively documented, showing positive effects on health improvement and weight gain of hosts (Gaggìa *et al.*, 2010). However, its use in aquatic organisms as growth enhancers has been more limited, and the report by Ringø *et al.* (2010) focused mainly on immune response and challenge trials. This study wanted to test their performance as promoters of growth and survival, as well as their effect on the modulation of gut microbiota in *C. humboldtianum* since these features have not yet been documented.

In particular, live food enriched with *L. johnsonii* C4 increased twofold the survival rate and final weight of the fingerlings of the shortfin silverside compared with the control group. Besides, a slightly but statistically significant amelioration in final length was recorded. Moreover, *B. animalis* subsp. *lactis* strain BB-12 bio-encapsulated into *A. franciscana* increase slightly survival rates, final weight, and final length. The increment on the survival rate of the shortfin silverside with *L. johnsonii* C4 application is supported by findings in the pike silverside *Chirostoma estor* (Jordan) in which larvae and juveniles that were fed with *L. casei* showed higher survival rates than the control group (Hernández-Martínez *et al*., 2009). Also, in the Nile tilapia *Oreochromis niloticus* survival rates were between 13-45% higher when a mixture of *Streptococcus faecium* and *L. acidophilus* or *S. cerevisiae* alone were used as a dietary supplement (Lara-Flores *et al*., 2003). These results suggest that *L. johnsonii* C4 could be an option to improve the rearing of the shortfin silverside. Further research must be done to verify and find out the mechanisms by which *L. johnsonii* C4 increase survival and fitness of the shortfin silverside. In contrast, the shortfin silverside growth was not promoted by *Bacillus* sp. B2 isolated from the freshwater angelfish, suggesting that a probiotic isolated from the gut of a fish might not be functional in other fish species. This idea is supported by the fact that most of the studies focused on the use of probiotics in aquaculture used autochthonous bacterial strains, but also commercial probiotic preparations and allochthonous strains have been successfully employed (Gatesoupe, 1999).

Immune system stimulation, inhibition of pathogenic bacteria colonization, and antimicrobial substances production are some suggested roles of probiotic bacteria (Martínez-Cruz *et al*., 2012). These traits enhance resistance against bacterial diseases and stress in early larval stages and could increase fish survival rates (Verschuere *et al*., 2000; Ringø *et al*., 2010). Probiotics can modify the population structure of bacterial communities associated with the alimentary canal of fish. Also, probiotics could modulate fish immune defenses, nutritional and growth performances, and water quality. The effect of gut microbiota

on fish fitness is a relatively new topic in aquaculture research (Gatesoupe, 1999; Balcazar *et al*., 2006a).

Fish intestinal microbiota is generally dominated by members of *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. The phylum *Proteobacteria* was predominant in the gut content microbiota of the shortfin silverside. The genera *Aeromonas* and *Vibrio* were found in the feces of the shortfin silverside by PCR-DGGE analysis. These genera were also identified in the anterior and posterior intestine of goldfish using the same technique (Silva *et al*., 2011). These taxa have frequently been retrieved from the gut of different fishes (Wu *et al*., 2012; Carda-Diéguez *et al*., 2014), and seem to be members of the gut bacterial community of healthy fishes. Even though some species of these genera may cause fish diseases (Sorroza *et al*., 2012; Talpur *et al*., 2014). In addition, the genus *Fusibacter* had been reported as a member of the gut bacterial community associated with the black tiger shrimp *Penaeus monodon*, but no function has been ascribed to this genus in the intestinal environment of aquatic animals (Chaiyapechara *et al*., 2012).

The genus *Exiguobacterium* detected by PCR-DGGE in the intestinal content of the shortfin silverside has been previously associated with *A. franciscana* (López-Cortés *et al*., 2006), and with the cultivable intestinal microbiota of the Atlantic salmon, *Salmo salar* (Linnaeus) (Ringø *et al*., 2008). However, to our knowledge, none ecological niche has been assigned to this bacterial genus in the fish gut environment. Some strains of *Exiguobacterium* can produce extracellular proteases (Kumar & Suresh, 2014), which could improve the functioning of the fish digestive system in larval stages.

PCR-DGGE patterns of 16S rRNA V3 and V6-V8 regions of intestinal bacterial communities associated with the shortfin silverside fed with different probiotics showed only slight changes at the end of the experiment. This result indicates that intestinal content bacterial community structure of the shortfin silverside is almost stable and well established after 21 days of hatching when we started the treatments with probiotics and is only slightly modified by the use of bacteria encapsulated in live prey. Similar results have been reported in the Atlantic halibut, *Hippoglossus hippoglossus* (Linnaeus) in which a steady gut bacterial community was obtained after three weeks of grazing with bacteria-treated live prey (Bjornsdottir *et al*., 2010). Also, in coho salmon, *Oncorhynchus kisutch* (Walbaum), the gut microbiota is established in early feeding stages, and it is initially influenced by aquatic environment and eggs epibiota (Romero & Navarrete, 2006). Although the gut bacterial community of the Shortfin Silverside seems to be established in early larval stages, an effect in fish fitness and survival could also be induced by the use of probiotic bacteria encapsulated in *A. franciscana* (Balcazar *et al*., 2008).

This study indicates that *L. johnsonii* C4 bio-encapsulated into *A. franciscana* could be used as probiotic in the shortfin silverside farming. This bacterium improved the growth and survival of the shortfin silverside. Use of probiotics bio-encapsulated into *A. franciscana* is a viable alternative to overcome the inability of the shortfin silverside juveniles to digest artificial food, which has been a bottleneck in the attempts to establish intensive aquaculture production of "pescado blanco". Future research should focus on testing the effectiveness of *Lactobacillus johnsonii* C4 bio-encapsulated into *A. franciscana* in intensive cultural practices of the shortfin silverside, with the final goal of recovering natural populations of this Mexican endemic fish and to create sustainable small-scale fisheries in rural communities.

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