Latin American Journal of Aquatic Research, 49(4): 551-564, 2021
DOI: 10.3856/vol49-issue4-fulltext-2672

Research Article

Lactobacillus strains isolated from oysters improve the production of *Crassostrea gigas* larvae

Macario Savin-Amador[1], Maurilia Rojas-Contreras[1], Pablo Misael Arce-Amézquita[1] Carlos Rangel-Dávalos[1] & Ricardo Vázquez-Juárez[2]
[1]Universidad Autónoma de Baja California Sur, La Paz, B.C.S. México
[2]Laboratorio de Genómica y Bioinformática, Centro de Investigaciones Biológicas del Noroeste Playa Palo de Santa Rita, México
Corresponding author: Maurilia Rojas-Contreras (mrojas@uabcs.mx)

ABSTRACT. One of the main problems in laboratories of oyster seed production occurs in the stage of settlement or fixation and metamorphosis of the larvae. The organisms develop drastic morphological and physiological changes at this stage, exposing them to attack by pathogenic microorganisms. Numerous studies indicate that before larval settlement to a substrate, they require bacteria to stimulate and induce the process. In this study, Japanese oyster (*Crassostrea gigas*) larvae were fed with combinations of microalgae and bacteria previously selected for their probiotic potential; their impact on survival, growth, and settlement was evaluated. Larvae D of 12 days old were provided by a private company and transported to the laboratory under controlled conditions. Probiotic bacteria *Lactobacillus plantarum* 69Cr, *L. fermentum* 101Cc, and *L. casei* 43Cg were reactivated on MRS and applied at a concentration of 1×10^4 CFU mL^{-1}. Microalgae *Isochrysis galbana* and *Chaetoceros calcitrans* were grown in F2 medium and supplied at a concentration of 3×10^4 cells mL^{-1}. Results showed, in all treatments, a survival range of 39-53%, growth of 300-310 µm, and 34-56.5% settlement. However, the best bacteria combined with *I. galbana* and *C. calcitrans* was *L. plantarum* 69Cr with a 54% survival, 310 µm growth, and 56.5% settlement. It is concluded that the combination of *L. plantarum* 69Cr with *I. galbana* and *C. calcitrans* has the potential to be used in the production of Japanese oyster larvae.

Keywords: *Crassostrea gigas*; larvae; *Lactobacillus plantarum*; settlement; probiotic; aquaculture

INTRODUCTION

Many sessile invertebrates, including Japanese oyster *Crassostrea gigas* (Thunberg, 1973), have larvae that freely swim until they are ready to undergo metamorphosis. Environmental signals, typically chemical, induce settlement and metamorphosis in almost all the studied systems (Burke 1983). It has also been reported that biofilms of some bacteria can induce the settlement of bivalve mollusks (Avendaño-Herrera et al. 2002, Alfaro et al. 2011, He et al. 2019).

One of the main problems in oyster seed production farms occurs at settlement or fixation and metamorphosis stages. During these processes, organisms develop drastic morphological and physiological changes (Illanes 1990), exposing them to the attack of pathogenic microorganisms. Throughout this period, they pass from a pelagic to benthic life. This change causes massive larval mortalities, reduced larval motility, erratic swimming, closing of the valves, detachment of the veil, and appearance of bacterial communities in and around the larvae (Beaz-Hidalgo et al. 2010, Rojas et al. 2015). Numerous studies have demonstrated the influence of biological, chemical, and physical factors on the induction of marine invertebrate larval settlement (Hadfield 2011, Huang et al. 2012, Salta et al. 2013). Other reports indicated that, before the settlement of the larvae to a substrate, biofilm-forming bacteria are required for settlement stimulation and induction (Chung et al. 2010, Yu et al. 2010, Wang et al. 2012, Yang et al. 2014). Biofilms are made up of diverse marine organisms; typically, they are composed

Corresponding editor: Sandra Bravo

of multiple bacterial species, which are the initial biological colonizers of new surfaces in the sea, along with diatoms, fungi, protozoa, and other microorganisms (Dobretsov 2010). Three bacterial biofilms of a single species have been reported to successfully induce metamorphosis in 100% of exposed coral *Acropora millepora* larvae (Tebben et al. 2011). Furthermore, it was also demonstrated that larvae of the mussel *Mytilus galloprovincialis* settled and metamorphosed in response to bacterial biofilms produced by the *Pseudoalteromonas* group (Satuito et al. 1995). Yang et al. (2013) concluded that the settlement and metamorphosis of *Mytilus coruscus* larvae were induced by some bacterial biofilms. *Pseudoalteromonas* bacteria have also been considered probiotics in aquaculture (Fjellheim et al. 2010, Kesarcodi-Watson et al. 2012, Newaj-Fyzul et al. 2014, Sorieul et al. 2018, Wang et al. 2018).

The word probiotic comes from the Greek, pro and bios (Schrezenmeir & De Vrese 2001). Various probiotic definitions have been presented since the first definition given by Lilly & Stillwell (1965), but the most widely used is the definition of World Health Organization (WHO): "Live microorganisms that, when administered in adequate amounts, confer a benefit for the health of the host". During the last decades, the benefits of probiotics in mollusk aquaculture have been described as growth promoters, enhancers of nutrition and environmental quality, immunostimulants, and prophylactic agents against infectious diseases (Cordero et al. 2014, Hoseinifar et al. 2018, 2019, Kuebutornye et al. 2019, Soltani et al. 2019). They have also been reported as a source of nutrients, vitamins, and digestive enzymes, which play an important role in food digestion, nutrient absorption, and growth (Lauriano et al. 2016, Nath et al. 2019).

However, there are few reports where probiotic bacteria have been used in mollusk larvae; in addition to protecting against pathogens, they stimulate settlement and produce biofilms. The objective of this study was to determine the impact of probiotic bacteria isolated from ostreids as inducers of the settlement of post-larvae of *C. gigas*, which can be used in massive trials in seed-producing laboratories.

MATERIALS AND METHODS

Strain reactivation

Probiotic strains used in this research were previously isolated from ostreids and characterized by their probiotic potential (Table 1). These strains are part of the bacteria collection stored at -85°C in the Food Science and Technology Laboratory (LABCyTA) at the Autonomous University of Baja California Sur (UABCS Spanish acronym). The strains were reactivated on MRS agar (Man, Rogosa and Sharp, DIFCO, Sigma Aldrich) by cross streaking and incubated in an anaerobiosis jar at 30°C for 48 h. Subsequently, each strain was grown on MRS broth (DIFCO) and incubated at 30°C for 12 to 18 h before use.

Larvae obtaining

Larvae were obtained using standard protocols in the private laboratory Marimex del Pacífico, S.A. de CV in La Paz, B.C.S., México, using mature broodstock from the environment. They were kept at a density of 20 larvae mL^{-1} in 500-L cylindrical tanks; a 1:1 mixture of *Isochrysis galbana* and *Chaetoceros calcitrans* was used as food at a ratio of 3×10^4 cells d^{-1} mL^{-1} (Helm et al. 2006). The larvae were placed in 1 µm filtered seawater (UV sterilized at 25 ± 1°C, salinity 37 ± 0.5) with constant aeration to maintain the culture.

Settlement test with probiotic bacteria

In this experiment, the three bacteria described in Table 1 and two-day-old larvae D (8 larvae mL^{-1}) were used. The larvae were placed in 20 L containers with 19 L of seawater, treated as mentioned above, with constant aeration. Treatments were carried out in triplicate as follows: 1) control treatment (without bacteria), 2) with *Lactobacillus fermentum* 101Cc, 3) with *Lactobacillus plantarum* 69Cr, and 4) with *Lactobacillus casei* 43Cg. The larval culture was performed with 100% water exchange every 24 h, adding the corresponding bacteria to each treatment after each exchange by immersion, at an effective dose of 1×10^4 CFU mL^{-1} (dose selected in the previous experiment, *unpubl. data*). The larvae were fed daily with a mixture of *I. galbana* and *C. calcitrans* (1:1) at a concentration of 3×10^4 cells mL^{-1}. Larval survival and condition state were determined in a phase-contrast microscope (Nikon Eclipse E-600) in three homogeneous samples of 1 mL per treatment per day. Survival was assessed by counting the normal swimming larvae and the dying larvae with closed valves and no veil movement. Condition state was evaluated according to Carreño et al. (2012), counting full larvae, showing an easily distinguishable dark brown to dark yellow digestive gland (DG), the half-filled ones with distinguishable light brown to light yellow DG, and the empty ones with translucent DG difficult to differentiate from the rest of the organs (Table 2). At the end of the experiment, 100 mL samples were randomly taken from each culture container to determine 30 larvae size. All observations were made using a phase-contrast microscope (Nikon

Table 1. Probiotic bacteria used in this study. BCS: Baja California Sur.

Strain	Genus	Species	Source	Tissue	Place
101Cc	*Lactobacillus*	*fermentum*	*C. corteziensis*	Digestive tract	BCS
69Cr	*Lactobacillus*	*plantarum*	*C. rhizophorae*	Digestive tract	BCS
43Cg	*Lactobacillus*	*casei*	*C. gigas*	Digestive tract	BCS

Table 2. Viable count of lactic acid bacteria in the treatments. *From day 12 to 14 in the treatment of crushed shells, there are no data because the eyespot began to appear on day 15.

Day/ Sample	Microbial viable counts								
	Water			Crushed shells			Wall		
	(log CFU mL^{-1})			(log CFU 0.2 g)			(log CFU cm^{-2})		
T	101Cc	69Cr	43Cg	101C	69Cr	43Cg	101Cc	69Cr	43Cg
12.00	3.36	3.36	3.30	*s/c	*s/c	*s/c	4.04	3.30	3.30
13.00	3.20	3.20	3.20	*s/c	*s/c	*s/c	4.26	3.30	3.00
14.00	3.20	3.41	3.48	*s/c	*s/c	*s/c	4.41	3.30	3.30
15.00	3.20	3.41	3.30	3.90	3.48	3.30	4.54	3.48	3.30
16.00	3.20	3.52	3.41	4.32	3.30	3.30	4.54	3.48	3.30
17.00	3.48	3.20	3.36	4.23	3.48	3.00	4.62	3.60	3.48
18.00	3.36	3.41	3.30	4.18	3.30	3.00	4.81	3.78	3.48
19.00	3.36	3.30	3.20	4.30	3.30	3.30	4.90	3.90	3.48
20.00	3.41	3.36	3.56	4.40	3.48	3.30	4.97	3.95	3.60
21.00	3.48	3.30	3.52	4.45	3.48	3.30	4.15	4.08	3.90

Eclipse E-600) and with a Sedgewick Rafter plate (SR) according to Lucas & Rangel's (1983) technique.

Oyster larvae settlement

After day 15th, the larvae already presented the "eye spot," verified through the microscope. They were placed at a density of 1 larva mL^{-1} in 20 L containers with seawater treated as described above with crushed shells prepared as follows: shells were crushed in a grain mill into small pieces and sieved with 250 and 120 µm meshes. Fragments between these two sizes were sterilized and added (2 g per container) to all treatments. Everyday samples (chunks) were taken from each tank in Petri boxes for size observation through a stereomicroscope (Labomed, model CxL (10x)). The larvae were kept there for six days before counting fixed, not fixed, alive and dead ones; and those that had biofilms on their exterior.

Microbiological analysis

Every day, a 1 mL sample of water was taken from each treatment, and viable counts were determined on MRS agar (DIFCO). Swab samples were also taken from the substrate (pieces of shell, approximately 0.2 g) and the walls (approximately 1 cm^2) to determine lactic acid bacteria with bacillary morphology.

Statistical analysis

Results were subjected to Barlett homoscedasticity test, and D'Agostino-Pearson's test for normality with an $\alpha = 0.05$ and then a one-way analysis of variance (ANOVA) was performed To compare survival, settlement, growth, and condition status between the treatments with the addition of probiotic bacteria and the respective controls. Determination of factors contributing to significant differences was carried out using the LSD multiple comparison test (Sokal & Rohlf 1980). Data collected as a percentage was transformed to arcsine before analysis.

RESULTS

Microbiological analysis showed that lactic acid bacteria (LAB) were present in water on average at 3.34, 3.34, and 3.38 log CFU mL^{-1} in the treatments 101Cc, 69Cr, and 43Cg, respectively, from day 12 to day 21. Also, LABs were present on the wall's containers at 4.04, 3.30, and 3.30 log CFU cm^{-2} in 101Cc, 69Cr, and 43Cg, respectively, on day 12. On day 21, they were present at 5.14, 4.00, and 3.90 log CFU cm^{-2} in 101Cc, 69Cr, and 43Cg, respectively. Analysis of shell pieces showed 3.90, 3.48, and at day 21, only changed treatment 101Cc with 4.45, log CFU/0.2 g (Table 2). In addition, a biofilm was observed in all treatments, except control (Fig. 5).

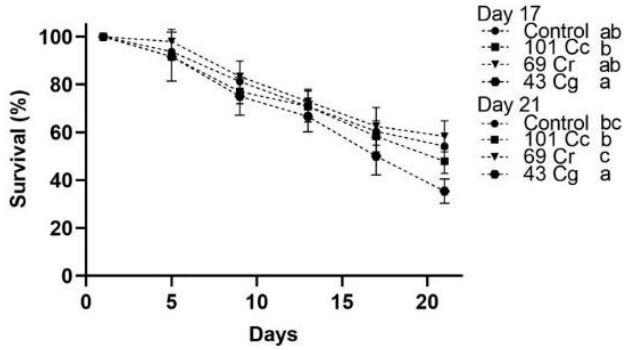


Figure 1. Survival from day 0 to 17 (before settlement) and at 21 days (after settlement) of *Crassostrea gigas* larvae treated with different probiotic bacteria. The data represents the mean ± standard deviation. Day 17 (a) in all treatments means no differences. Day 21, control (bc), 101Cc (b) and 69Cr (c) means no differences between them and significant differences compared to 43Cg (a) ($P > 0.05$) (Tukey HSD).

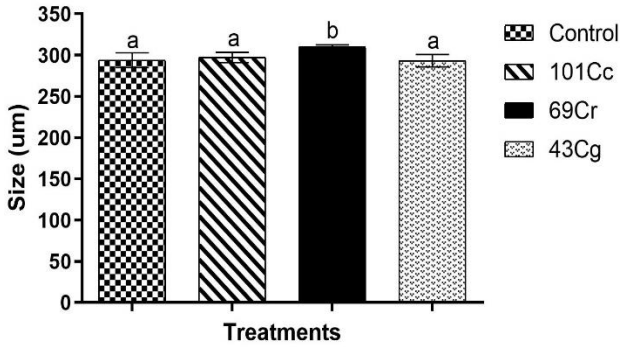


Figure 2. Size of the *Crassostrea gigas* larvae with different probiotic bacteria at day 21 after settlement on an artificial substrate. Identical letters denote lack of significant differences ($P > 0.05$). (a) In control, 101Cc and 43Cg means no differences between them, (b) in 69Cr means significative differences compared to (a).

Survival results did not show statistical differences ($P < 0.05$) between the treatments on day 16, before the larvae settlement (Fig. 1). On day 21, larvae survival in 101Cc, 69Cr, and control groups was higher and showed significant differences ($P < 0.05$) than 43Cg after settlement, however, 69Cr showed a higher percentage of survival (Fig. 1).

Growth results showed significant differences $P < 0.05$ on day 21, after settlement. Tukey's test showed that the larvae exposed to the 69Cr strain presented a significantly larger size ($P < 0.05$) than the control group, 101Cc, and 43Cg (Fig. 2).

Larvae condition was measured from day 14 to day 21. Data accumulated up to day 17, before settlement (Fig. 3), showed the predominance of full condition state in a range of 50.00 to 60.61%, followed by semi-full larvae in a range of 32.32-35.71%, and a small percentage of larvae in the empty condition in a range of 7.07-14.29%. It also showed significant differences between the 43Cg and 69Cr treatments *vs*. control. From day 17 to day 21, the daily condition state presented a higher percentage of full larvae and a lower percentage of empty and dead larvae (Fig. 4, Table 3). Bacteria biofilm on the surface of larvae was counted from day 12 to day 21 (Table 2) and observed from day 15 in all treatments except control by a confocal microscope (Laser Confocal Spectral Microscope, SP8 DMI8, LAICA; Objective 10X, software lasX) (Fig. 5).

Eyespot and settlement were evaluated directly under microscope observations, which showed that the eyespot was evident after day 12; however, statistical differences were observed after day 17, when a higher accumulated percentage of eyespot was observed (Fig. 6). A low percentage of settlement (<5%) was observed in all treatments after day 17, without statistical differences. After day 18, settlement increased above 30% in all treatments (Fig. 7), with statistical differences of 69Cr *vs.* 101Cc and control. By day 21, no larvae with eyespot were found, and only significant differences for settlement of 69Cr *vs.* 101Cc, 43Cg, and control were detected. The highest percentage (56%) of settled larvae was found in the 69Cr treatment (Fig. 7).

DISCUSSION

Probiotic strains used herein behaved differently during the development of *Crassostrea gigas* larvae, and only *Lactobacillus plantarum* 69Cr produced consistent results in all the parameters measured during the different stages studied, including settlement. Survival and growth of veliger larvae showed statistical differences compared to the control in the 69Cr treatment at the end of the experiment. Other bacterial strains used as probiotics in fish, mollusks, and crustaceans aquaculture, have shown their ability to improve host nutrients assimilation, growth, and resistance to diseases such as *Vibrio parahaemolyticus*, *Photobacterium damselae*, and *V. harveyi*, that improved water quality, and settlement of mollusk larvae (Silva et al. 2012, Abumourad et al. 2013, Abasolo-Pacheco et al. 2017, Correa et al. 2018, Nguyen et al. 2018, Pacheco-Vega et al. 2018, Shefat 2018, Huynh et al. 2019). Additionally, probiotic strains of this study, isolated from Ostreidae, characterized before as probiotic and applied to the same genus of organisms from which they were isolated, suggest a beneficial interaction between bacteria and host. Consistently, Campa-Córdova et al.

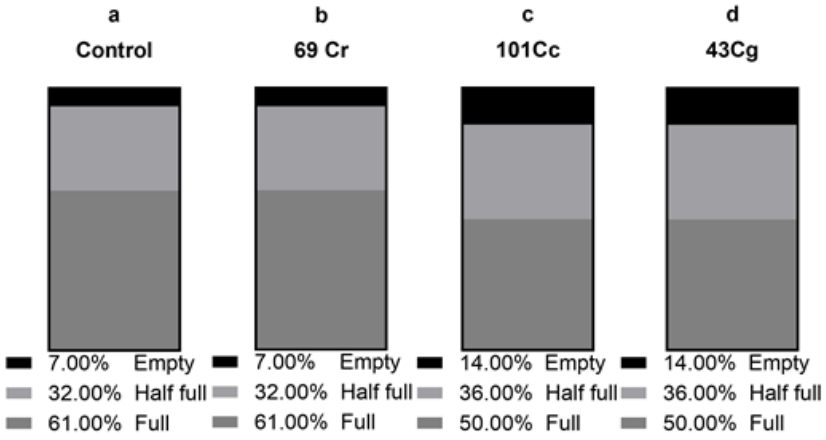


Figure 3. Condition status on day 17 before the settlement of *Crassostrea gigas* larvae fed with *Isochrysis galbana* and *Chaetoceros calcitrans* and administered with the different probiotic strains. a) Control (without bacteria), b) 69Cr (*Lactobacillus plantarum*), c) 101Cc (*L. fermentum*), d) 43Cg (*L. casei*).

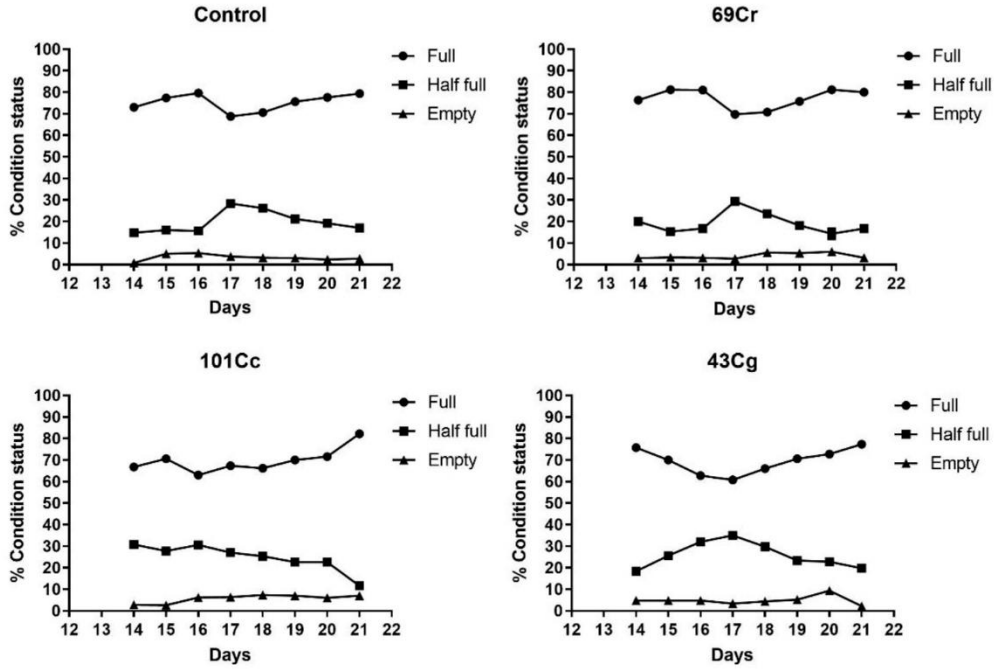
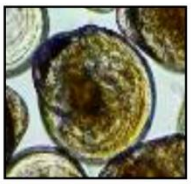
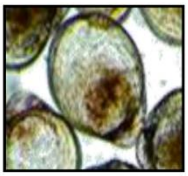
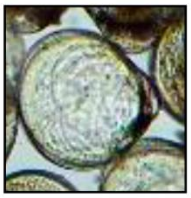


Figure 4. Daily condition status of *Crassostrea gigas* larvae fed with *Isochrysis galbana* and *Chaetoceros calcitrans* and administered with different probiotic strains. a) Control (without bacteria), b) 69Cr (*Lactobacillus plantarum*), c) 101Cc (*L. fermentum*), d) 43Cg (*L. casei*).

(2011), used preparations of *Lactobacillus* spp. isolated from lion paw clam *Nodipecten subnodosus* increasing growth and survival of laboratory-produced *Crassostrea corteziensis* larvae.

Larvae treated with the probiotic strains 69Cr, 101Cc and 43Cg presented a survival of 62, 58, and 50% respectively on day 17 without significant differences concerning the control. On day 21, 69Cr presented a higher percentage of survival concerning all treatments: control 54%, 101Cc 47%, and 43Cg 35%. These survival percentages are higher as compared to other investigations where the survival of *C. corteziensis* larvae was less than 50% when *Pseudomonas aeruginosa* and *Burkholderia cepacia* were added at concentrations of 0.1×10^3 to 10×10^3 CFU mL^{-1}. Also, survival was affected each time the concentration of the bacteria was increased (Campa-Córdova et al. 2011). Another study reported that the

Table 3. Larval condition status of *Crassostrea gigas* (Carreño et al. 2012).

Larva condition status	Description	Figure
Full	Digestive gland easily distinguishable, dark brown to dark yellow.	
Half full	Digestive gland distinguishable, light brown to light yellow.	
Empty	Transparent digestive gland, difficult to differentiate from the rest of the organs.	

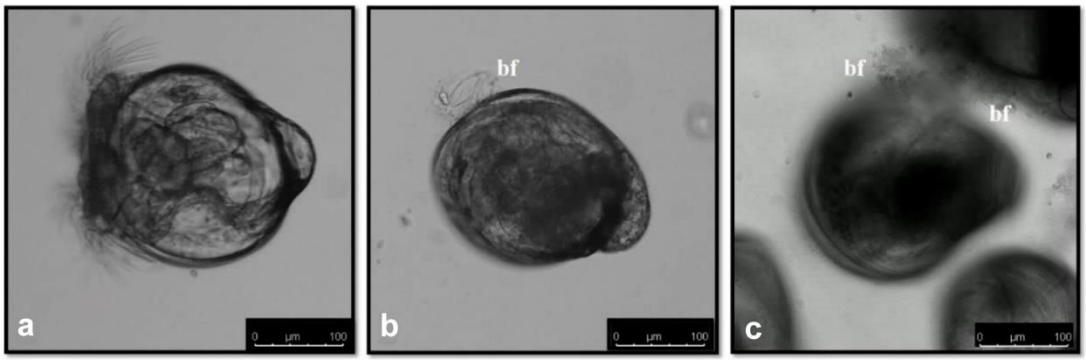


Figure 5. Larva veliger of *Crassostrea gigas* before settlement. a) Control treatment. Larva with open leaflets, no presence of biofilm; b) 69Cr treatment. Larva with semi-open leaflets, the biofilm (bf) can be observed on the periphery of the leaflets (Stereomicroscope Labomed, model CxL (10x)); c) 69Cr treatment. Larva with the presence of biofilm (Bf) (Microscopy Laser Confocal Spectral, SP8 DMI8, LAICA; Objective 10X, software lasX).

growth of *Perna canaliculus* larvae was significantly improved with a mixture of *Alteromonas macleodii* and *Neptunomonas* sp. at 10×10^6 CFU mL^{-1}, but the high rate of deaths occurred when 100×10^6 CFU mL^{-1} were added (Kesarcodi-Watson et al. 2012). Bacteria can be used as food for larvae of bivalve mollusks; for example, the marine bacterium strains CA2 consistently improved survival of *C. gigas* larvae around 21 to 22% in comparison with those fed only with the microalgae *Isochrysis galbana* (Douillet & Langdon 1993, 1994). Likewise, Catarina scallop *Argopecten ventricosus* larvae fed with strains PB1-1 (*Bacillus cereus*), PB1-6 (*Bacillus firmus*), and MIX-B resulted in significantly low survival values (16, 13, and 6% respectively) while using *L. plantarum* C3 increased survival above 40% (Abasolo-Pacheco et al. 2017). The effectiveness of *L. plantarum* as a probiotic has been reported in other marine organisms. In *Portunus pelagicus*, larviculture was investigated as a water additive and results indicated that *L. plantarum* addition at 5.0×10^6 CFU mL^{-1} can effectively enhance the larval survival rate and enzyme activity in the water quality (Talpur et al. 2013). Also, a diet supplemented with 10^{10} CFU kg^{-1} of *L. plantarum* upregulated prophe-

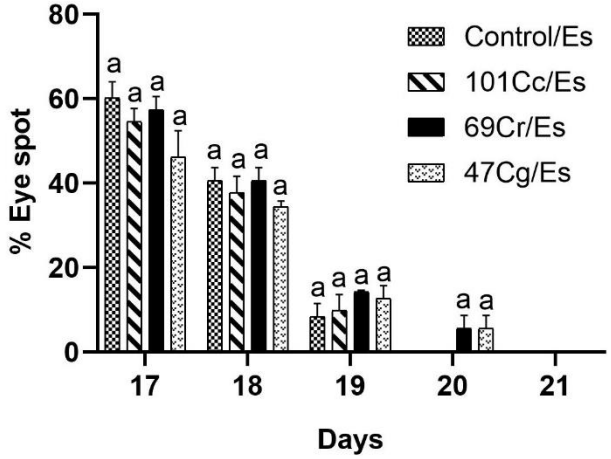


Figure 6. Eyespot incidence (Es) from day 17 to 21 in the larvae fed with *Isochrysis galbana* and *Chaetoceros calcitrans* and administered with different probiotic strains. Control (without bacteria), 69Cr (*Lactobacillus plantarum*), 101Cc (*L. fermentum*), 43Cg (*L. casei*). The data represents the mean ± standard deviation. (a) denote lack of significative differences between treatments per day ($P > 0.05$).

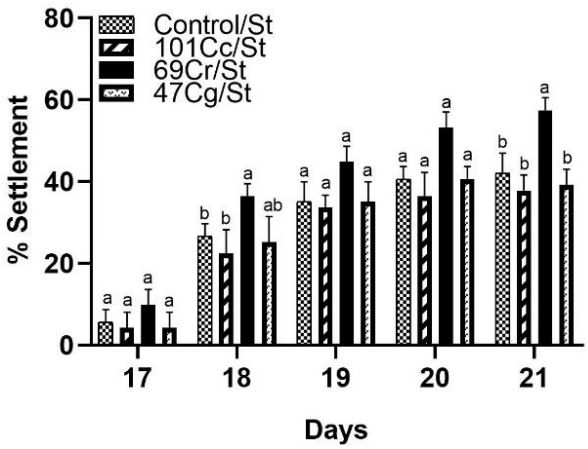


Figure 7. Cumulative settlement percentage (St) from day 17 to 21 of larvae fed with *Isochrysis galbana* and *Chaetoceros calcitrans* and administered with different probiotic strains. Control (without bacteria), 69Cr (*Lactobacillus plantarum*), 101Cc (*L. fermentum*), 43Cg (*L. casei*). The data represents the mean ± standard deviation. On days 17, 19, and 20, (a) denote no significant differences. In days 18 and 21 (a) means significant differences are compared to (b). ($P < 0.05$).

nol oxidase and phenoloxidase (PO) genes, PO superoxide dismutase activities, and resistance against *Vibrio alginolyticus* in *Penaeus vannamei* culture (Chiu et al. 2007). Similarly, it was reported that in *P. vannamei*, the diet supplemented with probiotic *L. plantarum* modulated intestinal microbiota as well as resistance against *V. harveyi* (Vieira et al. 2010). In addition, the probiotic effect of bacteria on hatchery-reared Catarina scallop early spat was reported. The spat treated with the yeast (S10) and *L. plantarum* (C3) significantly increased absolute growth and growth rate compared to the control (Vega et al. 2020).

On day 21, 43Cg (*L. casei*) presented the lowest percentage of survival, 35%. This species has been used as a probiotic in other marine organisms in higher concentrations than the concentration used in this report (effective dose of 1×10^4 CFU mL^{-1}). *L. casei* (0.7×10^8 CFU mL^{-1}), from the commercial product Yakult, was added to 50 mL of *Artemia cysts* for the enrichment and feeding to juvenile porthole livebearer *Poeciliopsis gracilis*; findings indicated that protein contents of skin mucus increased in fish fed with the nauplii enriched with *L. casei* but not improve the growth performance of *P. gracilis* (Hernandez et al. 2010). In another report, the effects of different levels of *L. casei*, used as probiotic, on growth performance and digestive enzymes activity of *Barbus grypus* was performed, and the results suggested that dietary supplementation of food with *L. casei* at 5×10^7 CFU g^{-1} concentration on day 30 and 5×10^8 CFU g^{-1} concentration on day 60 are suitable for enhancing the growth and digestive enzymes activity of *B. grypus* (Vand et al. 2014). *L. casei* isolated from rotifer and added at a density of 10^6 and 10^7 colony-forming units mL^{-1} protected *Artemia* against *Vibrio parahaemolyticus*. Strain *L. casei* X2 was selected as a candidate probiotic, due to the best growth performances of *Artemia*, with or without the pathogen (Lamari et al. 2014). The effect of probiotics *L. casei* YYL3 (Lc) and *L. plantarum* YYL5 (Lp) on channel catfish was performed, and results revealed that Lc, as a feed additive at 3.0×10^8 CFU g^{-1}, could promote growth performance, disease resistance and dramatically change the composition of the intestinal microbiota of this fish (Zhang et al. 2019). Regarding the effect of *L. casei* on the immune status of shabout, *Arabibarbus grypus*, a diet supplemented with different levels of *L. casei*, i.e. 5×10^6, 5×10^7, and 5×10^8 CFU g^{-1}, could likely enhance the immune responses and gene expression (Mohammadian et al. 2018). Comparing these reports with our results suggests that *L. casei* could be added in higher concentration to the larvae culture.

During the experiment, the larvae remained in the full condition state in all treatments, which indicates that they ingested food independently of the microalgae and the bacteria supplied. The former agrees with the data obtained by Lora-Vilchis & Maeda-Martínez (1997), who recorded that at least 70% of the larvae of the Catarina scallop *A. ventricosus* presented microalgal ingestion when testing ten microalgae species. In

this study, treatments given to *C. gigas* larvae maintained more than 80% intake, except on day 17, where ingestion decreased to 60 to 70%. This intake reduction could be related to the fixing process. Results also indicated that the 69Cr treatment (*L. plantarum* 69Cr + *I. galbana* + *C. calcitrans*) presented a significant increase in the percentage of full larvae after the settlement process. Probiotic bacteria can colonize the intestinal tract to improve digestion of microalgae (Soltani et al. 2019, Van Doan et al. 2019); also, they can produce growth factors and extracellular enzymes that strengthen the digestive and immune system of the host (Vine et al. 2006, Balcázar et al. 2008). Additionally, the treatments with the probiotic bacteria and the microalgae *I. galbana* and *C. calcitrans* showed a greater quantity of full larvae.

The settlement of mollusk larvae, according to several authors, is due to an induction mediated by natural biofilms or even induced biofilms (Prado et al. 2010, Wang et al. 2012, Yang et al. 2013, Peng et al. 2020). In this research, *Lactobacillus* probiotic strains formed biofilms on the walls of the tanks and the pieces of crushed shells. All treatments with the probiotic bacteria showed a settlement between 39 and 56%. Other authors have shown that these biofilms are bacterially produced and may positively influence the settlement and metamorphosis of marine invertebrates' larvae, including bivalve mollusks (Toupoint et al. 2012, Wang et al. 2012). The first complete studies about bivalve mollusks demonstrated the settlement of *Crassostrea virginica* and *C. gigas* larvae due to the biofilm induced by *Alteromonas colwelliana* LST, a pigmented marine bacterium, reaching a settlement response of 60 to 80%, proportional to the concentration of biofilms (Weiner et al. 1985, 1989).

Meanwhile, it was shown that *C. gigas* larvae treated with the bacteria *A. colwelliana*, *Vibrio cholerae* strain HTX, as well as non-pigmented bacteria *Escherichia coli* and *V. cholerae* strain 596-B, were settled in 30 and 90% in response to the supernatant of the culture media of these bacteria in the late logarithmic and stationary phase (Fitt et al. 1989).

Likewise, the biofilm formed by *Shewanella colwelliana* (Coyne et al. 1989) induced the settlement of *Ostrea edulis* larvae, but not of *Pecten maximus* (Tritar et al. 1992) Similarly, the effectiveness of the supernatant of the same strain to induce large-scale larval settlement of *C. gigas* and *C. virginica* in hatcheries was demonstrated (Walch et al. 1999). Other biofilms produced by a mixture of peripheral bacteria resulted in an *A. purpuratus* larvae settlement of around 25% (Avendaño-Herrera et al. 2002). Recently, Catarina scallop *A. ventricosus* larvae treated with *L. plantarum* C3 had a 2.38% success rate, while the control was 2.25% (Abasolo-Pacheco et al. 2017).

In this research, treatment with *L. plantarum* 69Cr resulted in 53% of the settlement. Although the chemical composition of biofilms formed was not investigated, *L. plantarum* has been widely studied for its ability to produce exopolysaccharides (EPS) with a variety of properties and activities, essential for its commercialization by food, cosmetic and pharmaceutical industries (Das et al. 2014, Fontana et al. 2015, Oh & Jung 2015, Zhang et al. 2016). EPS are composed of 90 to 96% carbohydrates and other substances such as sulfated compounds, protein, nucleic acids, and uronic acid (Wang et al. 2014, Imran et al. 2016, Zhang et al. 2016). EPS carbohydrates are non-toxic branched D-glucans with 86.5% α-(1→6) and 13.5% α-(1→3) bonds (Das et al. 2014). Additionally, *L. plantarum* strains have been recognized for their antimicrobial activity against biofilm-forming bacteria (Zhang et al. 2013, Li et al. 2014, Wang et al. 2015). It has been reported that EPS can mediate antimicrobial activity by modifying the surface of bacterial cells. This effect inhibits the initial adhesion of the bacteria to the surface or by acting with signaling molecules that regulate gene expression involved in biofilm formation (Kim & Kim 2009, Rendueles et al. 2013). The EPS of *L. fermentum* YW32 showed the ability to repress biofilm formation by Gram-negative and Gram-positive pathogens; however, the molecular mechanism is not yet elucidated (Wang et al. 2015).

In this investigation, larvae were treated with the probiotic strains 69Cr (*L. plantarum*), 101Cc (*L. fermentum*), and 47Cg (*L. casei*) showed a growth range of 294 to 310 µm until day 21 after settlement. In another report, where *C. gigas* larvae were fed with a marine bacterium, strain CA2, its growth was consistently improved (16 to 21%) compared to control cultures only fed with microalgae *I. galbana* (Douillet & Langdon 1993). The same authors, in another report, mentioned that bacteria concentrations above 10^7 cells mL^{-1} influenced the growth and survival of larvae, while concentrations ranging from 10^4 to 10^6 did not affect them (Douillet & Langdon 1994). *C. corteziensis* larval culture during 11 days with a mixture of strains of the *Bacillus* genus (1×10^4 CFU mL^{-1}) registered an average size of 189.5 µm, greater than those treated with strains of the genus *Lactobacillus* (169.9 µm) and that of the control without bacteria (169.6 µm), the growth was like the control at a concentration of 1×10^5 CFU mL^{-1} (Campa-Córdova et al. 2011). Likewise, *C. virginica* larvae added daily with the *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95 strains at a concentration of 10^4 CFU mL^{-1} were not affected in terms of growth or survival (Sohn et al. 2016).

The species *L. fermentum* and *L. casei* have been neither described in aquaculture for their ability to improve survival nor for growth and much less for bivalve mollusks larvae settlement. However, it has been reported that *L. fermentum* adheres to the intestinal mucosa of fish and reduces the adhesion of pathogens *in vitro* (Balcázar et al. 2008). It has been used in *P. vanammei* cultures for growth, survival, and resistance against *V. parahaemolyticus* (Sha et al. 2016a,b,c, Wang et al. 2019). Likewise, *L. fermentum* isolated from *Scomberomorus commerson* was able to inhibit the growth of *Listeria innocua* (Moosavi-Nasab et al. 2014). Similarly, *L. brevis* and *L. casei* have been used to decrease the load of *V. alginolyticus* in *Artemia* spp. culture (Villamil et al. 2003).

Recently, some microorganisms have been authorized in the European Union as probiotics in animal feed. Among those in the list of accepted microorganisms are *L. casei* and *L. plantarum* (EU, 2018).

According to traditional methods to produce bivalve mollusk seed, *I. galbana* y *C. calcitrans* are the most used microalgae (Helm et al. 2006). This study showed that probiotic bacteria and these microalgae presented a good performance, suggesting a positive interaction; however, other studies are required to understand that interaction.

CONCLUSIONS

As far as we could investigate, this is the first report where probiotic strains of the genus *Lactobacillus*, isolated from Ostreidae, contribute to the survival, growth, and settlement of *C. gigas* veliger larvae. The strains of the genus *Lactobacillus* isolated from ostreids and previously characterized by their probiotic potential in combination with *I. galbana* y *C. calcitrans* showed an important beneficial interaction with *C. gigas* larvae, so they are considered safe for use in the production of larvae of the Japanese oyster. Results showed high colonization and production of biofilm by the three strains (*L. plantarum* 69Cr, *L. fermentum* 101Cc, and *L. casei* 43Cg) on the walls of the tanks, shells (for settlement), and in the surrounding water. These strains contributed to the survival, growth, and settlement of the veliger larvae of *C. gigas*, being *L. plantarum* 69Cr the one that showed the best performance. In this way, the addition of probiotic strains of the genus *Lactobacillus*, isolated from ostreids, in the larval culture becomes a sustainable and reliable option to produce oyster seeds, particularly the combination of *L. plantarum* 69Cr with *I. galbana* and *C. calcitrans* which showed better results.

This research lays the foundations for more detailed studies, using molecular tools, on mechanisms of bacteria-larvae and bacteria-microalgae-larvae interaction, emphasizing the interactions of larvae with the native microbiota of the host and the microalgae used as food for the oyster.

ACKNOWLEDGMENTS

CONACYT Project #254648 supported this study. We thank CONACYT for financial assistance (Graduate fellowship 593764).

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Received: November 19, 2020; Accepted: March 3, 2021