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Antagonistic Interaction Between Beneficial and Pathogenic Bacteria Isolated from Ostreid Mollusks in Oyster Larvae (*Crassostrea gigas)*: An *In Vitro* and *In Vivo* Evaluation

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ABSTRACT

The Pacific oyster (*Crassostrea gigas* Thunberg, 1795) is a crucial species in global aquaculture; however, optimizing larval survival rates and ensuring high-quality seed production continue to pose significant challenges. Probiotics have been investigated as a potential solution throughout various stages of the production process. This study assessed strains of lactic acid bacteria (LAB) and pathogenic strains isolated from oysters. Notably, several LAB strains, particularly *Lactobacillus plantarum* 69Cr, demonstrated probiotic potential by inhibiting the growth of pathogens such as *Vibrio proteolyticus*. In Pacific oyster larvae, the *L. plantarum* 69Cr strain significantly improved survival, with an increase of over 80% compared to those exposed to the pathogen *Staphylococcus pasteuri*. These findings highlight the effectiveness of the *L. plantarum* 69Cr strain as a promising probiotic for protecting oyster larvae from pathogenic threats.

INTRODUCTION

Aquaculture continues to experience constant growth as an alternative to fishing, with the aim of increasing food production, generating jobs and opening new markets in various parts of the world, with annual increases of approximately 6.4% since 2001**(Nascimento-Schulze *et al*., 2021)**.

Pacific oyster, scientifically known as *Crassostrea gigas* (Thunberg, 1793), is a widely introduced species in various regions due to their exceptional tolerance and outstanding growth performance **(Ruesink *et al*., 2005; Miossec *et al*., 2009)**. This species is native to the western Pacific, from Sakhalin Island in Russia to the Yangtze River in

China **(Lodeiros *et al*., 2020)**. *C. gigas* is an estuarine species found in habitats ranging from solid bottoms, such as rocks, shells, and debris, to areas with mud and sand **(Helm *et al*., 2006)**. Its depth range extends from the mid-tidal area to 40 meters deep. This species is characterized by being extremely euryhaline, which means that it can tolerate wide variations in water salinity, ranging between 10 and 50 PSU (Practical Salinity Unit) and eurythermic, with a temperature tolerance ranging from -1.8 to 35°C. However, it is important to note that *C. gigas* reproduction occurs in a narrower thermal range **(Chavez-Villalba *et al*., 2003)**. *C. gigas* is a protandric hermaphrodite, meaning it can change sex throughout its life cycle. Females, which usually measure between 8 and 15cm, have the capacity to produce around 60 million eggs in a single spawning. In terms of growth, this species generally shows a rapid rate of development, managing in some regions to reach market size in less than 12 months of cultivation **(Lodeiros *et al*., 2018)**.

In the case of *C. gigas,* seed production is an activity over which there is total control. Since the late 1970s, oysters have been produced in numerous hatcheries (laboratories) around the world, which has made the seed widely available for introduction and trade, in order to support local, regional, national and even international aquaculture production. Global production of *C. gigas* reached 610,300 tons in 2020 **(FAO, 2022)**. Laboratories played a vital role in providing seed to expand and maintain this level of oyster production **(Nascimento-Schulze *et al*., 2021; FAO, 2022)**. One of the main difficulties in the commercial cultivation of marine organisms is the appearance of infectious diseases as a result of the incidence of bacteria, fungi and viruses frequently associated with the increase in culture densities, management methods, quality of water, nutritional value of the food, among other factors **(Lafferty, 2017; Baker-Austin *et al*., 2018)**. Even with adequate sanitary measures, outbreaks of diseases caused by opportunistic pathogens or bacterial infections continue to occur in laboratories of bivalve mollusks (oysters, clams, scallops, mussels). Moreover, vibriosis causes significant losses in laboratories that produce larvae and seedthat support bivalve aquaculture which is one of the most important sectors of aquaculture **(Dubert *et al*., 2017; FAO, 2018; Gradoville *et al*., 2018)**. Pathogenic species include *Vibrio aestuarianus, Vibrio coralliilyticus, Vibrio splendidus, Vibrio tapetis, Vibrio tasmaniensis and Vibrios tubiashii, Vibrio alginolyticus,* among others **(Elston *et al*., 2008; Richards *et al*., 2015; Dubert *et al*., 2017; Yang *et al*., 2021)**. Due to the high density of animals, it provides epidemiological and environmental conditions that give rise to possible outbreaks of infectious diseases that cause productive and economic losses.

In aquaculture, various strategies are used to mitigate mortality caused by bacterial diseases, such as antibiotic treatment and seawater disinfection. However, water disinfection can be expensive and, if done improper, can be harmful to larvae. Antibiotic treatment raises concerns both in the environmental field and in human health **(Prado *et al*., 2010; Baralla *et al*., 2021; Ferri *et al*., 2022)**. Therefore, the exploration of alternative approaches is essential to preserve an ideal environment for larval production and control

bacterial diseases in bivalve mollusk laboratories **(Yeh *et al*., 2020; Muñoz-Cerro *et al*., 2023)**.

An alternative to the use of antibiotics that is increasingly accepted in aquaculture consists of using probiotic bacteria to control microbial pathogens **(Van Doan *et al*., 2020; El-Saadony *et al*., 2021)**. Probiotics have been shown to be effective in preventing bacterial diseases in aquaculture **(Hoseinifar *et al*., 2018; Kuebutornye *et al*., 2019)**. Among the beneficial effects are increased growth, feeding efficiency and improved immune response **(Hardy *et al*., 2013; Hlordzi *et al*., 2020; Ringø., 2020)**. The term "probiotic" has evolved with various definitions over time. Initially, **Lilly and Stillwell (1965)** described it as a "substance produced by one microorganism that stimulates the growth of another." Later, **Fuller (1989)** proposed a more precise definition: "food supplement of live microorganisms that benefit the consumer by maintaining an adequate balance of the intestinal microbiota." These definitions have evolved based on the understanding and application of probiotics in different contexts. Currently, probiotics encompass various types of bacteria, bacteriophages, microalgae and yeasts that have been widely used in aquaculture, either through incorporation into water or as dietary supplements **(Bondad-Reantaso *et al*., 2023)**. Nowadays, numerous probiotics are available on the market both in the form of single strains and as microbial consortia **(Van Doan *et al*., 2017)**.

The fundamental probiotic property for application in aquaculture is antimicrobial activity, whose main purpose is to prevent and control diseases in aquaculture organisms through the inhibition of pathogens **(Balcázar *et al*., 2006; El-Saadony *et al*., 2021)**.

MATERIALS AND METHODS

1. Strains used in research

For this research, ten lactic acid bacteria (LAB) and ten pathogenic potential strains (PPS) were used, previously isolated from wild and cultured oysters from the coasts of the Mexican Pacific, along Baja California Sur state (BCS) and belonging to the microbial collection of the Food Science and Technology Laboratory (LABCYTA) of the Autonomous University of Baja California Sur (UABCS). The selected LAB strains were: 69Cr, 16Cc, 61Cg, 38Cg and 47Cg. These bacteria were previously characterized as Gram positive, catalase negative, with bacillary morphology **(Ramírez-Arroyo, 2013)** (Table 1). The PPS keys used were: O1, O3, O6, O11, O12, O18, O24, O62, O65 and O72 **(Sandoval, 2014)**. The following strains from the ATCC collection (The American Type Culture Collection) were also used: *Vibrio* sp. (25916), *V. proteolyticus* (15338), *Vibrio harveyi*, *V. parahaemolyticus* and *V. alginolyticus* (Table 1).

Table 1. Morphological and biochemical characteristics of the LAB and PPS used in this research

Lactic acid bacteria (LAB)	**Morphology**	**Gram stain**	**Oxidase test**	**Catalase test**
Pediococcus pentosaceus strain16Cc	*Bacillococcus*	Positive	Positive	Negative
Lactobacillus fermentum strain 38Cg	*Bacillus*	Positive	Positive	Negative
Lactobacillus plantarum strain 47Cg	*Bacillus*	Positive	Positive	Negative
Lactobacillus plantarum strain 61Cg	*Bacillus*	Positive	Positive	Negative
Lactobacillus plantarum strain 69Cr	*Bacillus*	Positive	Positive	Negative
Potentially Pathogenic Strains (PPS)				
Enterococcus sp. cepa O1	*Coccus*	Positive	Negative	Negative
Escherichia coli cepa O3	*Coccus*	Negative	Negative	Positive
Cronobacter sakazakii cepa O6	*Bacillus*	Negative	Positive	Positive
*Enterococcus faecalis cepa O*11	*Coccus*	Positive	Negative	Negative
*Staphylococcus pasteuri cepa O*12	*Coccus*	Positive	Negative	Positive
Escherichia coli cepa O18	*Bacillus*	Negative	Positive	Positive
Bacterium sp cepa O24	*Coccus*	Positive	Negative	Positive

Enterococcus faecium cepa O62	*Coccus*	Negative	Negative	Negative
Enterobacter cloacae O65	*Coccus*	Negative	Positive	Negative
Escherichia coli cepa O72	*Bacillus*	Negative	Positive	Positive
Collection strains				
V. parahaemolyticus	*Bacillus*	Negative	Positive	Positive
V. alginolyticus	*Bacillus*	Negative	Positive	Positive
Vibrio sp. (25916)	*Bacillus*	Negative	Positive	Positive
V. proteolyticus (15338)	*Bacillus*	Negative	Positive	Negative
V. harveyi	*Bacillus*	Negative	Positive	Negative

2. Plate diffusion assay

The antagonism assay was performed using the agar well diffusion method described by **Yilmaz *et al*. (2006)** and **Vinderola *et al*. (2008)** with some adjustments. The LAB strains previously selected for their probiotic potential (16Cc, 38Cg, 47Cg, 61Cg, 69Cr) were reactivated on MRS agar (DIFCO) by cross-striation and incubated in an anaerobiosis jar at a temperature of 30°C for 48 hours. Subsequently, the strains were reseeded in MRS broth (DIFCO) and incubated at 30°C for 12 to 18 hours before use. Drops of 5µL of each strain culture were inoculated, distributed in triplicate in each Petri dish with agar for standard methods, and incubated at 30°C for 48 hours. In parallel, strains with pathogenic potential were reactivated, a batch of each pathogenic strain was taken and seeded in 3mL of Trypto-Casein Soy Broth (TSB), and incubated at 35°C for 24 hours. Once the strains had grown, they were inoculated at 1% in malt agar (Standard Count Agar at a temperature of 40°C) and homogenized. Approximately 10ml of the inoculated culture medium was carefully poured onto each plate containing the probiotic bacteria colonies. About 10ml of the inoculated culture medium was carefully poured onto each plate containing the probiotic bacteria colonies. The agar was allowed to solidify and incubated at 30°C for 24 hours. The evaluation of the bactericidal effect was carried out by measuring the diameter of the inhibition zone (IZ) formed around the well, as described by **Jorgensen and Turnidge (2015**). The diameter of the IZ was measured in millimeters (mm) using a

vernier caliper, taking the measurement of the total diameter of the halo. The results of antibacterial activity were compared with positive controls.

3. Experimental system for *in vivo* selection of LAB and PPS in veliger larvae of *C. gigas*

An experimental system was installed in the mollusk area of UABCS, where independent experiments were carried out. Larvae were obtained following the standard protocols of **Helm *et al*. (2006)** in the mollusk laboratory of UABCS in La Paz, B.C.S., Mexico, using mature spawners from the environment. The larvae were maintained at a density of 20mL^{-1} in 100-liter cylindrical tanks using 1μm filtered seawater, sterilized by ultraviolet radiation at a temperature of 25± 1°C and a salinity of 37± 0.5, all under constant aeration. They were fed a 1:1 mixture of *Isochrysis galbana* and *Chaetoceros calcitrans* at a rate of 3×10^4 cells mL^{-1} day^{-1}, as indicated by **Helm *et al*. (2006)**.

4. Effect of LAB on the survival of *C. gigas* veliger larvae

In this experiment, five treatments were tested, each corresponding to a specific BAL: 16Cc, 38Cg, 61Cg, 69Cr, and 47Cg. The experiment was conducted in 3-liter containers filled with 2 liters of sterile filtered seawater at a concentration of approximately 8 larvae/mL. Each treatment had 3 replicates. Each probiotic bacteria strain was added to a container to reach a density of 1×10^7 CFU mL^{-1}. All containers were incubated for 48 hours at 25°C in a temperature-controlled room and fed with *I. galbana* microalgae at a concentration of 3×10^4 cells mL^{-1} day^{-1}.

5. In vivo selection of PPS for *C. gigas* larvae

The experiment was conducted in the same manner as the previous one. Veliger larvae were inoculated with the pathogenic strains listed in Table (1), with an administration dose of 10^7 CFU mL^{-1}**(Brown & Tettelbach, 1988)**. The CFU mL^{-1} of the required strains for each experiment was prepared using spectrophotometry based on the absorbance interpolated from a previously prepared standard curve.

6. In vivo challenge at pilot level of *C. gigas* veliger larvae administered with the BAL and selected PPS

Three of the 12 mentioned tanks were used, each with an operating volume of 100L of filtered seawater containing approximately 1.5 million veliger larvae. A cell suspension of the probiotic lactic acid bacteria selected in the previous experiment was applied. The bacterial suspension was prepared in flasks with 600mL of MRS broth inoculated at 1% from the reactivated culture. The suspension, approximately 1×10^9 CFU mL^{-1}, was applied at the beginning and after each water change, along with the microalgae, for 2 days. The control treatment was conducted in the same manner, but without adding of bacteria.

Another three tanks with an operating volume of 100L of filtered seawater containing approximately 1.5 million veliger larvae were treated with a cell suspension of the pathogenic bacteria selected in the previous experiment. The bacterial suspension was prepared in flasks with 600mL of Tryptic Soy Broth (TSB) inoculated at 1% from the pure culture. The suspension, 6×10^9 CFU mL^{-1}, was applied at the beginning and after each water change, along with the microalgae, for two days. In three additional tanks, each with an operating volume of 100L of filtered seawater containing approximately 1.5 million veliger larvae, a cell suspension of the probiotic bacteria (1×10^9 CFU mL^{-1}) previously isolated and selected from oysters was applied during the first two days of the experiment. On the third day, a cell suspension of the pathogenic bacteria (6×10^9 CFU mL^{-1}) was added. This culture was also applied after water changes and throughout feeding. The organisms with the pathogen were maintained for 2 more days and then sacrificed for analysis. The final three tanks, each with an operating volume of 100L of filtered seawater containing approximately 1.5 million veliger larvae, served as a control, with no bacteria added. Survival was determined by observing the internal structure of the larvae under a phase contrast microscope (Nikon Eclipse E-600). Deterioration or retraction of internal organ structures or lack of food intake was taken as indicative of dead or dying larvae.

7. Statistical analysis

The results were subjected to Bartlett's test for homoscedasticity and the D'Agostino-Pearson normality test with an $\alpha = 0.05$. Subsequently, an analysis of variance (ANOVA) was conducted to compare the larval survival rates between BAL and PPS. The determination of factors contributing to significant differences was carried out using the LSD multiple comparison test **(Sokal & Rohlf, 1980)**. Data collected as percentages were transformed using arcsine before analysis. All analyses were performed using GraphPad Prism version 8.4.3 software.

RESULTS

Plate diffusion assay

The results of the inhibition test are presented in Table (2). Only the combinations where there were inhibition zones are shown. In the analysis of the lactic acid bacteria (LAB) evaluated, the *L. plantarum* 69Cr strain stood out, which exhibited a strong inhibitory capacity against *V. proteolyticus* (15338) with an inhibition diameter of 13.2mm. This antimicrobial activity was also evidenced against *V. alginolyticus* (12.9mm), *V. parahaemolyticus* (12.4mm), *E. coli* (O18) (8.6mm), *E. coli* (O3) (7mm) and *S. pasteuri* (6.3mm), reflecting the effectiveness of *L. plantarum* 69Cr against various strains. Strain 61Cg also showed notable inhibitory activity, standing out with an inhibition diameter of 9.6mm against *E. coli*, 8.3mm against *V. harveyi*, 7.5mm against *V. proteolyticus* (15338), 9.6mm against *E. coli* (O3) and 7.8mm against *V. parahaemolyticus*. On the other hand,

strain 47Cg exhibited an inhibitory capacity with a halo of 10.3mm against *V. harveyi*, 9.6mm against *E. coli* (O18), 5.3mm against *E. coli* (O3) and 3.5mm against *V. parahaemolyticus*.

Table 2. Strains with probiotic potential showing antagonism against potentially pathogenic bacteria

LAB key	Genus and species	PPS Key	Genus and species	Inhibition zones (mm)
47Cg	*L. plantarum*	O3	*E. coli*	5.3
47Cg	*L. plantarum*	O18	*E. coli*	9.6
61Cg	*L. plantarum*	O18	*E. coli*	5.6
61Cg	*L. plantarum*	O3	*E. coli*	9.6
69Cr	*L. plantarum*	O3	*E. coli*	7
69Cr	*L. plantarum*	O12	*S. pasteuri*	6.3
69Cr	*L. plantarum*	O18	*E. coli*	8.6
47Cg	*L. plantarum*	*V. parahaemolyticus*	*V. parahaemolyticus*	3.5
47Cg	*L. plantarum*	*V. proteolyticus* (15338)	*V. proteolyticus* (15338)	4.2
47Cg	*L. plantarum*	*V. harveyi*	*V. harveyi*	10.3
61Cg	*L. plantarum*	*V. parahaemolyticus*	*V. parahaemolyticus*	7.8
61Cg	*L. plantarum*	*V. proteolyticus* (15338)	*V. proteolyticus* (15338)	7.5
61Cg	*L. plantarum*	*V. harveyi*	*V. harveyi*	8.3
69Cr	*L. plantarum*	*V. parahaemolyticus*	*V. parahaemolyticus*	12.4

69Cr	*L. plantarum*	*V. proteolyticus* (15338)	*V. proteolyticus* (15338)	13.2
69Cr	*L. plantarum*	*V. alginolyticus*	*V. parahaemolyticus*	12.9

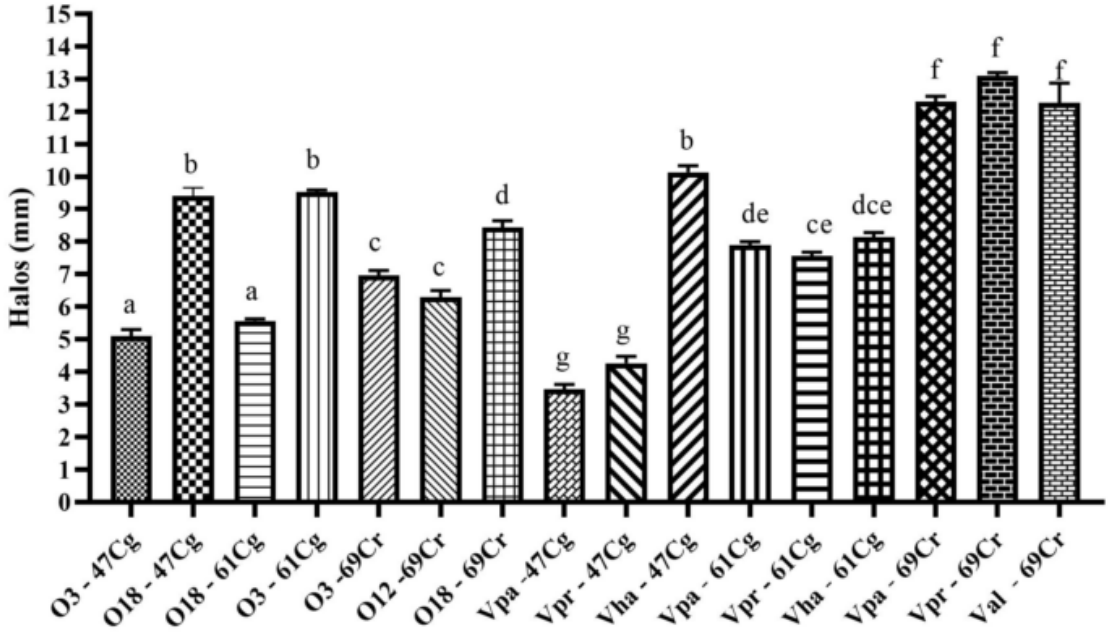

Fig. 1. The bars indicate the millimeters of the inhibition zones of LAB against PPS and collection pathogens. Significant differences are observed (ANOVA $P< 0.05$) between the strains with probiotic potential and the pathogens used. Three homogeneous groups were identified through multiple comparison tests of the least significant difference, and for treatments with identical letters, there were no significant differences (ANOVA $P< 0.05$). Vpa: *Vibrio parahaemolyticus*, Vpr: *Vibrio proteolyticus*, Vha: *Vibrio harveyi*, Val: *Vibrio alginolyticus*

Significant differences were observed between the treatments of the PPS strains, specifically in the cases of O3-47Cg, O3-61Cg, O3-69Cr, O18-47Cg, O18-61Cg, O18-69Cr, O12-69Cr. Likewise, significant differences were recorded in the collection strains Vpa-47Cg, Vpr-47Cg, Vha-47Cg, Vpa-69Cr, Vpr-69Cr and Val-69Cr. However, no significant differences were found in the Vpa-61Cg, Vpr-61Cg and Vha-61Cg treatments.

Effect of the probiotic on the survival of the veliger larvae of *C. gigas*

Of the seven LAB probiotic strains evaluated with oyster veliger larvae over eight days, only three demonstrated significantly higher larval survival than the control group. Meanwhile, another three maintained similar survival levels; one exhibited lower survival than the control group. Fig.(2) illustrates the survival of *C. gigas* veliger larvae cultured for eight days with different LAB strains, all at a concentration of 1×10^7 CFU mL^{-1}. On the sixth day, larval survival was significantly higher in treatments with *L. plantarum* 69Cr and *L. fermentum* 38Cg (Tukey, $P<0.05$) than those treated with other strains and the control group. Conversely, treatments with strains *L. plantarum* 101Cc and *L. fermentum* 47Cg showed lower survival levels than the control group, although these differences were not statistically significant.

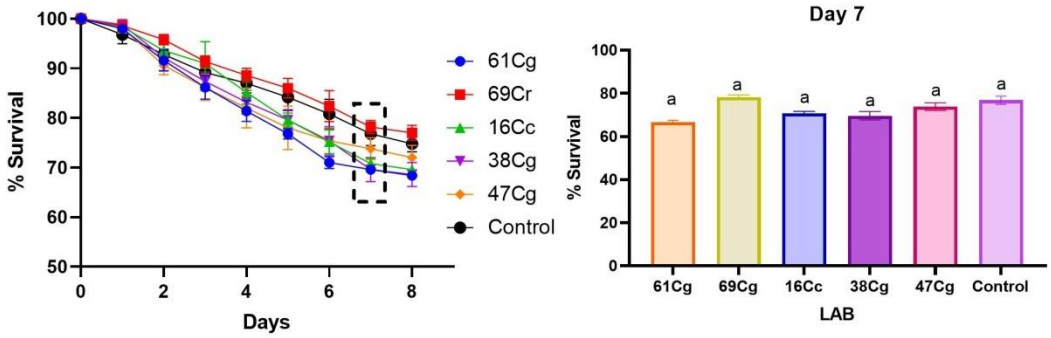

Fig. 2. The survival of veliger larvae treated with different probiotic strains over eight days of testing. Significant differences are observed between treatments with different letters, while no differences are found between treatments with the same letters ($P< 0.05$)

Survival of *C. gigas* veliger larvae with PPS *in vivo*

Among the ten PPS and collection strains administered to oyster veliger larvae over the eight-day (Table 1), most resulted in complete mortality by the fourth day of exposure. However, strain O12 and *V. parahaemolyticus* exhibited a different pattern, causing total larval mortality from the second day onward, as illustrated in Fig. (3).

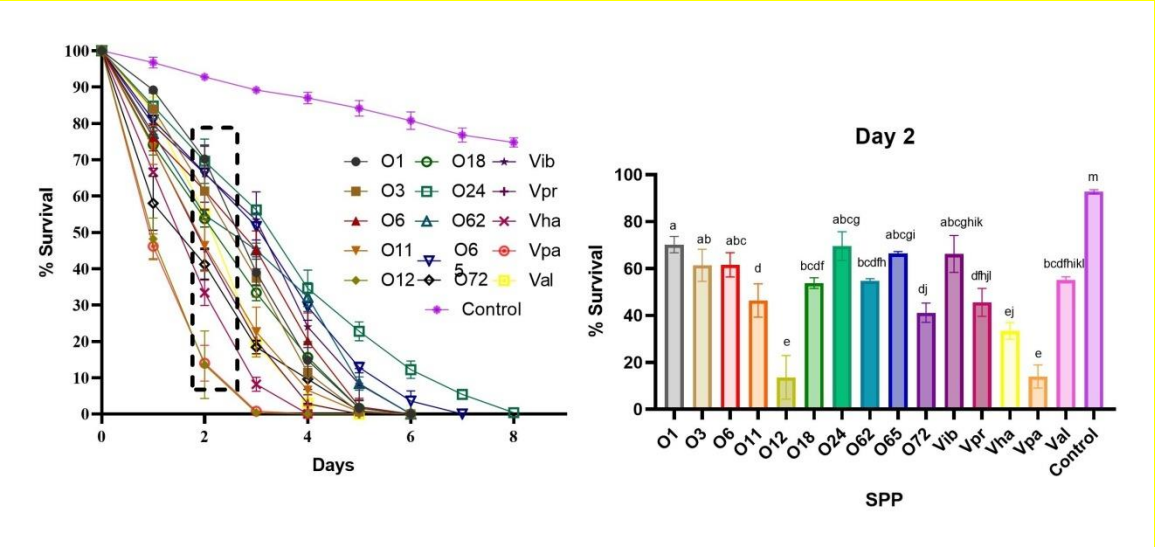

Fig. 3. The survival of *C. gigas* veliger larvae cultured for eight days with different pathogenic strains at a concentration of $1x10^7$ CFU mL^{-1} is shown. Larval survival was very low from the second day of exposure to the bacteria. According to the Tukey test ($P< 0.05$), no significant differences were observed between treatments on day seven

***In vivo* challenge of *C. gigas* veliger larvae with probiotic *L. plantarum* 69Cr against pathogen *S. pasteuri* O12**

This study, evaluated the *in vivo* efficacy of the lactic acid bacterium (LAB) *L. plantarum* 69Cr in protecting veliger larvae from the pathogenic strain *S. pasteuri* O12. The experimental protocol involved initially treating the larvae with the probiotic bacteria, followed by the introduction of the pathogenic bacteria two days later. After this period, the probiotic administration was discontinued, and the larvae were maintained with only the pathogenic strain for an additional two days before being sacrificed and placed in vials for subsequent analysis. As shown in Fig. (4), the results demonstrate a probiotic effect from the first day of treatment, with a significant improvement in the survival of larvae treated with the probiotic. Furthermore, even after introducing the pathogenic bacteria, larvae maintained significantly higher survival rates than the control group, highlighting the protective capacity of LAB *L. plantarum* 69Cr in the presence of the pathogen.

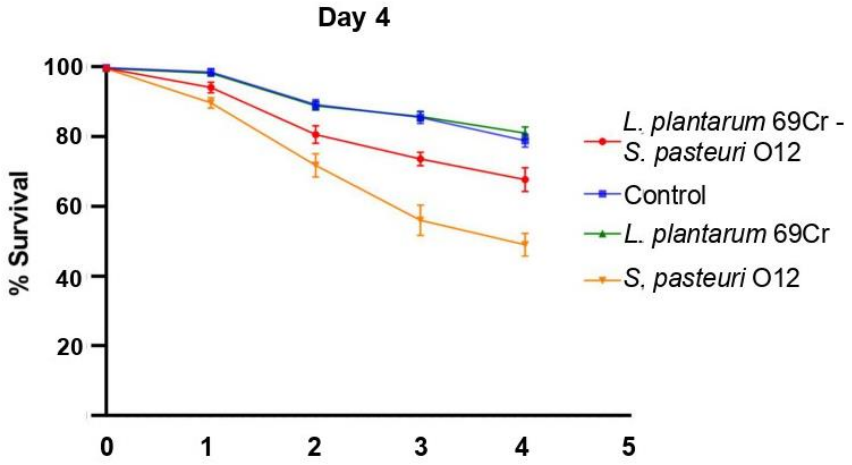

Fig. 4. Survival of *C. gigas* veliger larvae cultured for five days with the BAL 69Cr *L. plantarum* strain, PPS *S. pasteuri* O12, and their combination, each at a concentration of $1x10^7$ CFU mL^{-1}

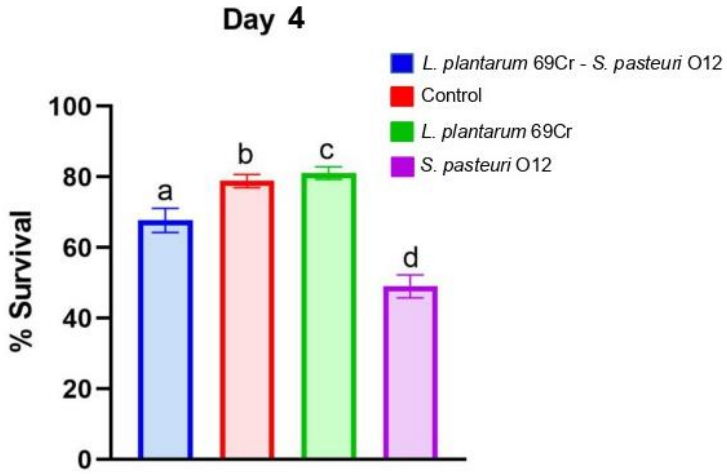

Fig. 5. Survival of *C. gigas* larvae with the selected LAB, selected PPS, their combination, and the control treatment on day seven. Different letters denote significant differences ($P< 0.05$): (a) *L. plantarum* 69Cr – *S. pasteuri* O12 treatment, (b) control, (c) *L. plantarum* 69Cr, and (d) *S. pasteuri* O12

Fig. (4) shows the percentage of survival of 2-day-old veliger larvae on the 1st and 2nd days of exposure to the probiotic strain *L. plantarum* 69Cr and the 3rd and 4th days of exposure to the pathogenic bacteria *S. pasteuri* O12, compared to the control treatment. Fig. (5) reveals that on day 1, the survival percentage of veliger larvae treated with the probiotic strain was significantly higher than the control ($P= 0.01787$). On day 2, no

significant differences were observed (P= 0.08023). On day 3, after introducing the pathogenic bacteria, the survival percentage of the veliger larvae remained significantly higher compared to the control (P= 0.03951). On day 4, with the continued addition of the pathogenic bacteria, the survival percentage was very significantly higher than the control (P= 0.0004232), with an $\alpha = 0.05$.

DISCUSSION

Plate diffusion assay

The antagonistic capacity of LAB is a crucial factor in their interaction with pathogenic bacteria. **Borges *et al*. (2021)** highlighted that these bacteria can secrete antagonistic compounds and/or digestive enzymes, enabling them to compete effectively for space and nutrients. This property can be evaluated *in vitro* through antagonism assays against various pathogens of interest. Furthermore, **Alvarez-Sieiro *et al*. (2016)** emphasized that the substances produced by these bacteria include organic acids, such as lactic acid and acetic acid, diacetyl, ethanol, hydrogen peroxide, and bacteriocins. *In vitro* antagonism assays were conducted to evaluate the inhibitory effects of LAB using the plaque diffusion assay method. The trials targeted ten PPSs and five collection pathogens. The results indicated that LAB exhibited antagonistic effects against PPSs and collection pathogens. Among the strains tested, *L. plantarum* strains 47Cg, 61Cg, and 69Cr produced well-defined inhibition halos. Notably, strain 69Cr demonstrated the highest average inhibition zone.

Evidence from previous research supports the effectiveness of various bacterial strains in inhibiting relevant pathogens in aquaculture. In line with these findings, **Porsby *et al*. (2016)** demonstrated that *Phaeobacter inhibens* DSM 17395 exhibits antagonistic properties against *Vibrio vulnificus*, suggesting its potential to limit the establishment of this pathogen in adult oysters within a model system. Similarly, **Nair *et al*. (2021)** tested antagonism of *Bacillus subtilis* Ba37 against seven major aquaculture pathogens. The results revealed significant antibacterial activity, particularly against *V. vulnificus* MTCC1145 and *V. harveyi*, with less pronounced effects against *Aeromonas hydrophila* and *Aeromonas veronii*, highlighting the selective capacity of this strain in targeting specific pathogens.

The study by **Escamilla-Montes *et al*. (2023)** provides new information on specific strains in relation to bacterial activity. It was evident that the MT4H2 *Bacillus* strain exhibits activity against *V. parahaemolyticus* IPNGVE16. In contrast, the BAL strain used in the study did not demonstrate activity against the same pathogen. Furthermore, it was observed that the isolate MT4H2 (*Bacillus*) exhibited antagonist activity against *V. parahaemolyticus*. At the same time, MT1E2 (BAL) did not show the ability to inhibit its growth in the antagonism assay in plates.

Probiotic effect on the survival of *C. gigas* veliger larvae

The use of probiotics in aquaculture has evolved significantly from early artisanal methods where probiotics were isolated and cultivated on individual shrimp farms **(Rodríguez *et al*., 2007)** to the availability of commercial products such as Sanolife® MIC and PrimaLac® **(Decamp *et al*., 2008; Miandare *et al*., 2016)**. Probiotics have emerged as highly effective agents for disease control in aquatic invertebrate culture, particularly in Asia and South America. This evolution underscores the role of probiotics as powerful tools in disease management empowering the aquaculture community with effective solutions **(Sharifuzzaman *et al*., 2022)**.

In aquaculture, the use of various microorganisms as probiotics, particularly bacteria from the *Bacillus* and *Lactobacillus* genera, has been widely observed **(Ringø, 2020)**. *L. plantarum* strains have been isolated from the oysters **(Ramirez-Aroyo, 2013; Khouadja *et al*., 2017)**, shrimp **(Correa *et al*., 2018; Zheng *et al*., 2020)**, and intestinal mucosa of several fish species **(Alonso *et al*., 2019; Valipour *et al*., 2019; Ruizhe *et al*., 2023)**. These studies indicate that *L. plantarum* produces growth inhibitory factors against various pathogenic *Vibrio* species. **Alonso *et al*. (2019)** demonstrated the inhibition of *V. harveyi*, *Vibrio splendidus*, and *Photobacterium damselae* using *L. plantarum* in fish culture water. Similarly, **Ruizhe *et al.* (2023)** utilized *Lactobacillus plantarum* (now classified as *Lactiplantibacillus plantarum* according to **Zheng *et al.* (2020)**) to inhibit the growth of *Vibrio alginolyticus* (MCCC 1A03220), *Vibrio harveyi* (MCCC 1A03227), *Vibrio campbellii* (MCCC 1A08161), and *Aeromonas hydrophila* (MCCC 1A00007).

The results of this research indicate that *L. plantarum* 69Cr, a bacterium found in the marine environment, colonizes the digestive system of oysters and, compared to other species, stimulates larval growth and settlement **(Savin-Amador *et al*., 2021)**. This bacterium establishes a positive interaction with its host and exhibits antagonistic effects against *Staphylococcus pasteuri*. Moreover, *L. plantarum* 299v has been shown *in vitro* to possess an antimicrobial activity against potentially pathogenic species such as *Vibrio ichthyoenteri*, *Edwardsiella tarda*, *Streptococcus iniae*, and *V.parahaemolyticus* **(Kang *et al*., 2016)**. *L. plantarum* 69Cr also displays relatively strong antagonistic properties against *Salmonella enterica* subsp. *enterica* and intermediate antagonistic activity against *Helicobacter pylori* **(Hütt *et al*., 2006)**. *Helicobacter pylori* is a significant public health concern due to its association with conditions such as peptic ulcer disease (PUD) and gastric cancer, with a global prevalence estimated at 50%, and reaching up to 90% in some countries **(Goh *et al*., 2011; Hooi *et al*., 2017)**.

C. gigas larvae exhibited significantly longer survival rates when treated with *L. plantarum*. In contrast, the poorest survival was observed in larvae treated with other LAB strains compared to the control group. Relatively few studies have investigated the effects of marine LAB on bivalve larvae. For instance, in other aquatic species, such as the largemouth bass (*Micropterus salmoides*), *L. plantarum* strains have been shown to improve digestion & protein absorption and to reduce the proportion of pathogenic bacteria

in the intestine **(Yang *et al*., 2023)**. Similarly, **Abdel-Latif *et al*. (2023**) reported that feeding fry of the freshwater fish *Pangasianodon hypophthalmus* with a diet supplemented with *L. plantarum* enhanced their defense against infectious bacterial pathogens. **Tseng *et al*. (2023)** demonstrated that supplementing the white shrimp (*Penaeus vannamei*) with *L. plantarum* improved immune response, antimicrobial activities, and resistance to *V. alginolyticus* infection. In the bivalve *Aequipecten opercularis*, the *L. plantarum* strain was found to increase growth **(Čanak *et al*., 2023)**. Additionally, using the *P. inhibens* S4 strain in *Crassostrea virginica* larvae controlled vibriosis and reduced mortality **(Takyi *et al*., 2023)**. **Freire-Peñaherrera *et al*. (2020)** utilized the *Bacillus amyloliquefaciens* A5 strain to inhibit *V. vulnificus* growth *in vitro*, and this strain was also effective in colonizing live larvae and preventing *V. vulnificus* colonization. **de la vega-vega *et al*. (2020)** reported that *L. plantarum* significantly increased the shell height of *Argopecten ventricosus* compared to the control group.

Similarly, **Abasolo-Pacheco *et al*. (2017)** observed a significant increase in the size and weight of *A. ventricosus* juveniles when supplemented with *L. plantarum*. **Savin-Amador *et al*. (2021)** demonstrated that the *L. plantarum* strain enhanced the survival and attachment rates of *C. gigas* larvae. **Madison *et al*. (2022)** evaluated the *Vibrio coralliilyticus* RE22 strain in *C. gigas* larvae, administering probiotic bacteria at a concentration of 3×10^4 CFU mL^{-1}. The candidate strains DM14 and B1 showed a 68% increase in relative percentage survival (RPS) compared to the positive control *V. coralliilyticus*, with high average survival rates of 99.71 ± 0.87 and 96.29 ± 3.30%, respectively. In this context, **Amador *et al*. (2021)** demonstrated that *C. gigas* larvae fed with *L. plantarum* at a concentration of $1x\ 10^4$ CFU mL^{-1} and exposed to *V. parahaemolyticus* at $3.14\ x\ 10^5$ CFU mL^{-1} showed an improved survival. The larvae exhibited a high overexpression of genes related to the immune system, such as heat shock proteins (HSPs), actins, and dual oxidase. In this study, the survival rate with the *L.plantarum* 69Cr strain was 80%. Even when the larvae were exposed to the pathogen, their survival rate remained between 60-70% compared to other studies.

S. pasteuri is commonly found in food, as well as in the air and on surfaces **(Santoiemma *et al*., 2020)**. While *S. pasteuri* has not been documented as a pathogen for oysters, this study revealed that it caused mortality in oyster larvae, primarily within two days of exposure. Surprisingly, this strain was the least inhibited in the antagonism test. Meanwhile, the *L. plantarum* 69Cr strain exhibited a high inhibitory effect against the selected pathogens, and the larvae that were administered with these bacteria showed a higher survival level.

CONCLUSION

The specific study of the *S. pasteuri* strain in this context revealed unexpected results. Although this strain had not previously been classified as pathogenic for oysters, it was observed to cause larval mortality, especially during the two days after exposure.

Notably, the *S. pasteuri* strain was the least inhibited in the antagonism test. In contrast, the 69Cr *L. plantarum* strain exhibited a prominent inhibitory effect despite not being commonly associated with pathogenicity in oysters. These findings underline the importance of thoroughly examining the specific interaction between bacterial strains and host organisms in aquaculture. The ability of some strains, such as *L. plantarum*, to improve larval survival in *C. gigas* larvae contrasts with the negative impact observed with the *S. pasteuri* strain. The variability in results highlights the need to carefully select probiotic strains to maximize their benefits in different aquaculture contexts.

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