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Growth, survival, and superoxide dismutase activity in juvenile *Crassostrea corteziensis* (Hertlein, 1951) treated with probiotics

Crecimiento, supervivencia y actividad superoxido dismutasa en juveniles de *Crassostrea corteziensis* (Hertlein, 1951) tratados con probióticos

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ABSTRACT

Juvenile seed of the Cortés oyster *Crassostrea corteziensis* were exposed to *Lactobacillus* sp. isolated from *Nodipecten subnodosus*, a mix of *Pseudomonas* sp. and *Burkholderia cepacia*, a marine yeast strain, a commercial probiotic (Epicin®), and oxytetracycline to determine their effect on growth, survival, SOD activity, and protein content. Probiotics at the test dose of 50,000 cells·ml^{-1}, Epicin and oxytetracycline at 7 mg·l^{-1} were evaluated during 30 days of culture. Results showed that growth of *C. corteziensis* was significantly improved by *Lactobacillus* sp. and the bacilli mix significantly enhanced survival and SOD activity at the test dose. Protein content did not significantly increase by the treatments used. This study demonstrated the potential use of marine microbiota to improve cultivation of *C. corteziensis.*

Key words: Probiotics, *Crassostrea corteziensis*, SOD, survival, growth.

RESUMEN

Juveniles de Ostión de Cortés *Crassostrea corteziensis* fueron expuestos a *Lactobacillus* sp., aislado de *Nodipecten subnodosus*, una mezcla compuesta de *Pseudomonas* sp. y *Burkholderia cepacia*, una levadura marina, un probiótico comercial (Epicin®) y oxitetraciclina, para determinar su efecto en el crecimiento, supervivencia, actividad superóxido dismutasa (SOD) y contenido de proteína. Los probióticos fueron utilizados a una concentración de 50,000 cells·ml^{-1}, el Epicin y la oxitetraciclina a 7 mg·l^{-1} y sus efectos se evaluaron durante 30 días de cultivo. Los resultados mostraron crecimiento significativo de *C. corteziensis* con *Lactobacillus* sp. e incremento significativo en supervivencia y actividad SOD con la mezcla de bacilos. El contenido proteico no registró incremento significativo con los tratamientos utilizados. Este estudio muestra el uso potencial de la microbiota benéfica aislada de invertebrados marinos para mejorar el cultivo de *C. corteziensis.*

Palabras clave: Probióticos, *Crassostrea corteziensis*, SOD, supervivencia, crecimiento.

INTRODUCTION

Bivalve mollusk culture is a profitable economic activity worldwide. Cultivation of filter-feeding bivalves is one of the potential and sustainable forms of aquaculture that can be operated on a large scale with no artificial food because bivalves can obtain nutrients from phytoplankton, microphytobenthos, and organic detritus (Hawkins *et al.*, 2001). Cultivation of bivalves is also

useful for reducing fishing effort of wild native species (Pipitone *et al.*, 2000). The Cortés oyster *Crassostrea corteziensis* (Hertlein, 1951) inhabits the Pacific coast from the Gulf of California to Panama (Keen, 1971) and is a suitable candidate for commercial cultivation. Like other bivalves species, cultivation of *C. corteziensis* has several problems that need to be addressed. One of the main problems is high mortality during larval and juvenile culture, largely caused by bacteria. *Vibrio* sp., have been recognized as pathogenic for bivalves, including *Crassostrea virginica* (Gmelin, 1791) (Elston & Leibovitz, 1980), *C. gigas* (Thunberg, 1793) (Sugumar *et al.*, 1998), *Argopecten purpuratus* (Lamarck, 1819) (Riquelme *et al.*, 1995), *Pecten maximus* (Linnaeus, 1758) (Lambert *et al.*, 1999), *Ruditapes philippinarum* (Adams & Reeve, 1850) (Borrego *et al*,. 1996), *Argopecten ventricosus* (Sowerby II, 1842) (Luna-González *et al.*, 2002), *Nodipecten subnodosus* (Sowerby I, 1835) (Luna-González *et al.*, 2002), and *Atrina maura* (Sowerby, 1835) (Luna-González *et al.*, 2002).

Apart from good cultivation practices, antibiotic supplements are used to prevent mortality of larvae and juvenile bivalve species (Luna-González *et al.*, 2004). However, there is widespread concern that antibacterial agents in aquaculture lead to the emergence of resistant bacteria (Scholz, 1996).

Probiotic treatment has been successfully carried out in mollusks (Macey & Coney, 2005), fish (Robertson *et al.*, 2000; Brunt *et al.*, 2007), and crustacean species (Harzevili *et al.*, 1998; Rengpipat *et al.*, 2000; Rodríguez *et al.*, 2007). Probiotics used in aquaculture studies include Gram-positive and Gram-negative bacteria, bacteriophages, yeast, and unicellular algae (Irianto & Austin, 2002). Beneficial effects include growth and feed efficiencies (Venkat *et al.*, 2004). Studies demonstrated control of *Vibrio tubiashii* infections in *Crassostrea gigas* larvae (Gibson *et al.*, 1998), inhibition of *Vibrio* sp. that enhanced survival of *Pecten maximus* larvae (Ruíz-Ponte *et al.*, 1999) and *Argopecten purpuratus* larvae (Riquelme *et al.*, 2000), and improvement of growth and resistance to disease in *Haliotis midae* (Linnaeus, 1758) (Macey & Coney, 2005).

The complex antioxidant system of aerobic organisms prevents the effect of reactive oxygen species (ROS), and also protects cells from oxidative stress (Downs *et al.*, 2001). Enzymatic antioxidant defenses include ascorbate peroxidase, glutathione reductase, catalase, peroxidases, and superoxide dismutase (SOD), which scavenges the superoxide anion (Homblad & Söderhall, 1999). SOD plays an important role in modulating oxidative responses leading to increased or decreased SOD activity (Matsuda *et al.*, 2003).

A common way to select probiotics is to perform *in vitro* antagonism tests, in which pathogens are exposed to candidate probiotics in a liquid or solid medium (Balcázar *et al.*, 2006). It is essential to document the origin, safety, and ability of the strain to survive the transit through the gastrointestinal tract of the host (Gram *et al.*, 2001).

This study reports the *in vivo* effect of three bacteria species, one marine yeast strain, a commercial probiotic formulation, and a commercial antibiotic on growth, survival, and antioxidant response in *C. corteziensis* seed. Attention is paid to cellular SOD (Matsuda *et al.*, 2003; Li *et al.*, 2005), which plays an important role in modulating oxidative responses.

MATERIALS AND METHODS

Maintenance and feeding of specimens. Healthy juvenile *C. corteziensis* (shell length 0.82 ± 0.1 mm) were maintained at the hatchery of Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, Mexico, in small polyethylene tanks containing 4-l filtered (1-mm), and aerated seawater at 25 ± 1 °C and salinity of 36 ‰. Seed were acclimated for 3 days and fed 1.5×10^5 cells·ml^{-1} of a mixture of *Isochrysis galbana* Parke, *Chaetoceros calcitrans* (Paulsen) Takana, and *C. gracilis* Schütt (1:1:2) before the treatments.

Bacterial strains and culture conditions. Bacterial strains were previously isolated from the intestine tract of adult lions-paw scallop *Nodipecten subnodosus* collected from Bahía de La Paz Baja California Sur, Mexico (~24.3°N, ~110.3°W), from the intestinal tract of adult whiteleg shrimp (*Litopenaeus vannamei*, Boone, 1931) cultured in a shrimp farm near La Paz, B.C.S., and from the intestinal tract of adult *C. corteziensis* from an oyster farm in the State of Baja California Sur, Mexico. Bacterial strains were selected from in vitro antagonism tests against pathogenic bacteria (*Vibrio alginolyticus* and *V. harveyi*), and from hemolytic activity tests (using bovine erythrocytes). Strains were stored in specific medium (MRS medium, or YPD medium) supplemented with 15% glycerol at –80 °C until used. Bacterial strains were identified using the BIOLOG system.

Lactobacillus strain NS6.1, isolated from *Nodipecten subnodosus*, was incubated in MRS agar medium at 30 °C for 24 h. *Pseudomonas aeruginosa* strain YC58, isolated from *Litopenaeus vannamei* and *Burkholderia cepacia* strain Y021, isolated from C. *corteziensis*, were incubated in YPD agar medium at 30 °C for 24 h, blended in a 1:1 ratio (Mix). The marine yeast *Yarrowia lipolytica* strain 020 was obtained from the collection at CIBNOR, selected because of its in vitro antagonistic activity (against the pathogen bacteria *V. alginolyticus* and *V. parahaemolyticus*) and lack of hemolytic activity (using bovine erythrocytes), and cultured in YPD agar medium at 30 °C for 24 h. A commercial probiotic formulation, Epicin®, (Epicore Bionetworks, Mount Holly, NJ, USA) was tested, as was oxytetracycline (Sigma, #Cat. O4636) as an antibiotic.

Preparation of probiotics for *C. corteziensis*. Probiotics were thawed and incubated in specific medium at 30 °C for 24 or

48 h. Cells were removed from the culture medium by centrifugation (14,000 x g, 5 min, 4 °C) and resuspended in 3% sterile saline solution at a final concentration of 1×10^9 CFU·ml^{-1} (stock concentration). The concentration of probiotics in the *C. corteziensis* culture container was adjusted from the stock concentration. A stock solution was prepared for Epicin and oxytetracycline treatments, adjusted to a concentration of 7 mg·l^{-1} in seed culture from both stock solutions.

Experimental protocol. Groups of 50 juveniles were cultured in 4 L plastic containers with 1 µm-filtered and aerated seawater at 25 ± 1 °C and salinity of 36‰. Culture tank water was changed totally every 48 h. Seeds were fed daily with 3×10^5 cells·ml^{-1} of *Isochrysis galbana*, *Chaetoceros calcitrans*, and *C. gracilis* (1:1:2). Triplicate groups of juveniles were treated with lactobacilli/ bacilli (Mix), or yeast at 5×10^4 CFU·ml^{-1}, and with oxytetracycline or Epicin at 7 mg·l^{-1} for 30 days. A triplicate control group was cultured in filtered seawater free of any treatment. Temperature and salinity were measured daily. The concentration of treatment ingredients in the containers was restored with every seawater change. Survival and growth were recorded at day 30. Six juveniles from each container were randomly sampled for protein content and SOD activity and stored at –80 °C.

SOD extraction and activity assay. For cell disruption, 0.1 g frozen tissue was removed from seeds and added to a mechanical homogenizer containing 0.5 ml phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged at 5,724 x g for 5 min at 4 °C (Beckman model GS-15R; Rotor No. F2402). The supernatant was recovered and heated for 5 min at 65 °C. A new supernatant was obtained after a second centrifugation (crude extract) and stored at –20 °C.

SOD activity was determined according to Beauchamp and Fridovich (1971), using nitro blue tetrazolium (NBT) in the presence of riboflavin. Briefly, 2 ml reaction mixture (0.1 mM EDTA, 13 µM methionine, 0.75 mM NBT, and 20 µM riboflavin added to 50 mM phosphate buffer at pH 7.8) and 0 to 100 µl crude extract were placed under fluorescent light for 2 min or until A_{560} in control tubes reached 0.2 to 0.25 OD. SOD activity (units per milligram protein) was calculated using a computer program (Vázquez-Juárez *et al.*, 1993).

Protein determination. Total soluble protein concentration in juvenile *C. corteziensis* (from 100 mg tissue) was measured according to Bradford (1976), using bovine serum albumin as a standard. Protein content was expressed in mg·ml^{-1}.

Statistical analysis. One-way ANOVA using Statistica 6.0 software (StatSoft, Tulsa, OK, USA) was used to analyze the difference between treatments and controls. Values of $p < 0.05$ were considered significantly different. When significant differences were found, Tukey's HSD test, using Statistica software was used to identify the significance of these differences ($p < 0.05$).

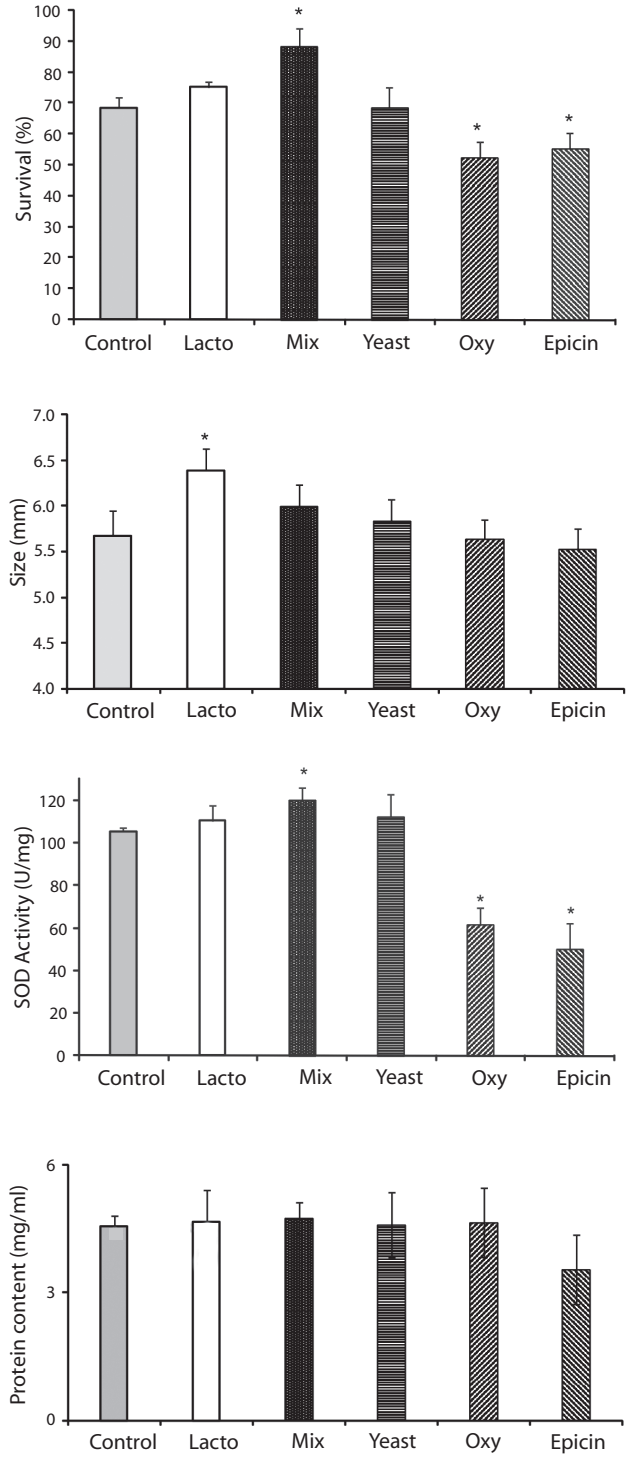

Figure. 1a-d. Effects of treatments with probiotics on juvenile *Crassostrea corteziensis* seed during a 30-day period. a) Percentage of survival. b) Growth rate. c) SOD activity. d) Protein concentration. Treatments were: Lacto = *Lactobacillus* sp., strain NS6.1. Mix = *P. aeruginosa*, strain YC58, + *B. cepacia*, strain Y021, 1:1 ratio. Yeast = *Y. lipolytica*, strain 020. Oxy = oxytetracycline. Epicin = commercial probiotic. Data are expressed as mean ± SD. * = Significantly different than control ($p < 0.05$).

RESULTS

Survival. Juveniles treated with bacilli (Mix) had significantly ($p < 0.05$) higher survival than the control group (Fig. 1). Survival was significantly ($p < 0.05$) lower than the control group after exposure to oxytetracycline or Epicin.

Growth. Figure 2 shows growth of juvenile *C. corteziensis* exposed to various treatments for 30 days. Juveniles exposed to *Lactobacillus* sp. (NS6.1) showed significantly ($p < 0.05$) more growth rate than the control group, whereas, juveniles treated with the commercial probiotic (Epicin) had the least growth of all treatments.

SOD activity. Juvenile *C. corteziensis* exposed to *Lactobacillus* sp. or yeast cells for 30 days did not differ significantly in SOD activity (Fig. 3) compared with the control group. However, seed treated with the bacteria Mix showed significantly ($p < 0.05$) greater SOD activity (120.27 $U \cdot mg^{-1}$) than the control group (105.25 $U \cdot mg^{-1}$). Juveniles exposed to oxytetracycline, and Epicin had significantly lower SOD activity (Fig. 3).

Protein. Protein concentration of treated *C. corteziensis* was not significantly ($p > 0.05$) increased over than the control group (Fig. 4) and seed treated with Epicin had the lowest concentration of protein.

DISCUSSION

In an intensive aquatic production system, disease control plays a key role, where an intimate relationship between bacteria and host is present. Probiotics have proven advantageous in domestic animal production and the evidence supports the same conclusion for microbial management in rearing aquatic animals (Carnevali *et al.*, 2004; Rodríguez *et al.*, 2007). Probiotics can be delivered directly to the water via live carriers, such as *Artemia salina* (Linnaeus, 1758) nauplii and rotifers, or added to pelleted dry feed (Gomez-Gil *et al.*, 2000). Only a few studies have focused on bacteria that prevent the growth of pathogenic organisms in aquaculture systems (Harzevilli *et al.*, 1998; Kesarcodi-Watson *et al.*, 2008). Vijayan *et al.* (2006) suggested using probiotic bacteria to inhibit the growth of bacterial mollusk pathogens.

Pathogenic *Vibrio* cause large die-offs during larval and grow-out phases of mollusks (Vijayan *et al.*, 2006). For at least two decades, prophylactic and therapeutic use of antibiotics has been practiced in commercial hatcheries (Gatesoupe, 1989), but this appears to have let to antibiotic resistance (Sahul Hameed *et al.*, 2003). Antibiotics commonly used in aquaculture are oxytetracycline, furazolidone, chloramphenicol, erythromycin, streptomycin, kanamycin, neomycin, and oxolinic acid (Benbrook, 2002). Campa-Córdova *et al.* (2005) reported higher larval survival in *Argopecten ventricosus* treated with 6.0 $mg \cdot l^{-1}$ of chloramphenicol and erythromycin. Our results showed that 7 $mg \cdot l^{-1}$ of oxytetracycline did not enhance growth, survival, antioxidant activity, or protein content in juvenile *C. corteziensis*.

Frequently, lactic acid bacteria have been used as probiotics (Carnevali *et al.*, 2004; Rengpipat *et al.*, 2008). In our study, the use of *Lactobacillus* sp. at 5×10^4 $CFU \cdot ml^{-1}$ enhanced growth in juvenile *C. corteziensis*. These bacteria often produce bacteriocins and other chemical compounds that inhibit the growth of pathogen bacteria (Gildberg *et al.*, 1997; Goldschmidt-Clermont *et al.*, 2008), and induce higher growth and feed efficiency (Venkat *et al.*, 2004). *Lactobacillus* spp. have been reported to provide benefits to human health, such as reducing cholesterol, absorption of nutrients, promoting lactose digestion, ameliorating gastrointestinal microflora, producing some vitamins, preventing some cancer, viral infection, and allergies, and having an immuno-modulatory effect (Kawahara & Otani, 2006). Bacteria used in this study may provide essential nutrients not present in algae or improved feed digestion by contributing enzymes (Verschuere *et al.*, 2000). Moal *et al.* (1996) reported that bacteria in the gut of bivalve larvae consist of many strains that produce intracellular enzymes, including proteases and lipases.

Pseudomonas spp. are common inhabitants of soil, freshwater, and marine environments and are known to produce a wide range of secondary metabolites, such as antibiotics, hydrogen cyanide, or iron-chelating siderophores, and inhibit a wide range of pathogenic bacteria. *Pseudomonas* spp. and *Vibrio* spp. are the most common genera associated with aquatic environments (Otta *et al.*, 1999). Chythanya *et al.* (2002) indicated that the antagonistic action that inhibits vibriosis is pyocyanin, a chloroform-soluble substance. Gram *et al.* (1999) observed in vitro inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* and lower mortality in the probiotic-treated fish *Oncorhynchus mykiss* (Walbaum, 1792). Specific inhibition of *V. harveyi* by *Pseudomonas aeruginosa* was reported by Torrento and Torres (1996). Riquelme *et al.* (2001) observed increased survival in *Argopecten purpuratus* larvae fed with *a Bacillus* sp.

Having an open circulation system, bivalve mollusks ingest biotic and abiotic particles, including pathogens from the surrounding water (Allam & Paillard, 1998). If bacteria or other pathogenic microbes enter the body of an invertebrate, a series of immune defense reactions will normally be elicited (Cheng, 1978). One of these defense reactions is the toxic reactive oxygen intermediates formed during a respiratory burst and which play an essential role to clear invading pathogens from the shellfish tissue and hemolymph (Mitta & Vandenbulcke, 2000). If the oxidant/antioxidant balance is an important determinant of immune cell function, increased levels of antioxidants will be needed to improve the immune response. SOD eliminates superoxide free radicals and plays an important role in protection against oxidative stress (Leclère, 2004). Gonzalez and Arenas (2002) concluded that SOD activity and production of the superoxide anion in *A. purpuratus*

hemocytes could be used to evaluate the competence of the immune system in mollusks. In our study, seed treated for 30 days with live bacilli mix, significantly increased SOD activity compared to the control group. Decreased antioxidant activity in seed treated with oxytetracycline, and Epicin may induce oxidative stress in *C. corteziensis* according to Shuhong *et al*. (2004). They exposed adult *Haliotis diversicolor supertexta* (Reeve, 1846) to *Escherichia coli* and *Vibrio* spp. and found a significant decreased SOD activity in treated groups compared with controls.

In our study, protein content did not show significant ($p > 0.05$) variation in *C. corteziensis* exposed to probiotics, but other studies have related protein content to immune response in invertebrates. Downs *et al*. (2001) related increased protein content after exposure to immunostimulants to the protective effect of the immune system in grass shrimp, *Palaemonetes pugio* (Holthuis, 1941), against potential pathogens. Campa-Córdova *et al*. (2002) found a significant increase in protein content in *Litopenaeus vannamei* hemocytes after exposure to 0.5 $mg \cdot ml^{-1}$ of ß-glucans.

Yeasts have been reported as promising probiotics (Vine *et al*., 2006., Macey & Coney, 2006). Shupantharika *et al*. (2003) reported significantly enhanced phenoloxidase activity of hemocytes of the giant tiger prawn *Penaeus monodon* (Fabricius, 1798) treated with brewer's yeast ß-glucan in vitro and in vivo. In contrast to these studies, the marine yeast (*Yarrowia lipolytica* strain 020), used in one of our treatments, did not induce a significant increase in growth, survival, or physiological response. This marine yeast species has been reported to incorporate exogenous eicosapentaenoic and docosahexaenoic fatty acids from crude fish oil (Guo *et al*., 1999).

What these results of treating juvenile *C. corteziensis* with probiotics show is that some beneficial bacteria increase shellfish well being as indicated by enhanced growth and survival. Further research should provide information to optimize the concentration of probiotics in the diet of juvenile *C. corteziensis*. We also recommend that specific cells or tissues, especially hemocytes be used to evaluate antioxidant activity and immune response.

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