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Growth and antioxidant response of juvenile oysters *Crassostrea sikamea* and *Crassostrea corteziensis* treated with *Streptomyces* strains

[*Crescimento e resposta oxidative de ostras jovens* Crassostrea sikamea *e* Crassostrea corteziensis *tratadas com cultura* Streptomyces]

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ABSTRACT

The effect of three *Streptomyces* strains (N7, RL8 and V4) and a mixture of *Bacillus* (BMix) on the growth (Weight, Size) and superoxide dismutase activity (SOD) in hatchery-reared juvenile oysters *Crassostrea corteziensis* and *Crassostrea sikamea* was investigated to determine their probiotic potential. Microorganisms were added to culture water at 1×10^6 CFU/ml once a day during 30 days and all oysters fed daily a microalgae mix. Juveniles of *C. sikamea* treated with strains N7, RL8 and V4 had a significant weight gain compared to the control group. *C. corteziensis* juveniles treated with strains RL8 and BMix showed a significantly higher weight gain than the control group. No significant size increase was observed in any treated group for both oyster species. SOD activity significantly increased in C. *sikamea* treated with RL8 and with RL8, N7 and BMix in *C. corteziensis*. *Streptomyces* strains RL8 and N7 emerge as promising probiotic agents to cultivate *C. sikamea* and *C. corteziensis* and may also be useful to other molluscs and marine invertebrates**.**

Keywords: actinomycetes, oyster, growth, probiotic effect, SOD activity

RESUMO

O efeito de três culturas Streptomyces *(N7, RL8 e V4) e uma mistura de* Bacillus *(BMix) sobre o cresimento (Peso, Tamanho) e atividade superóxido dismutase (SOD) em ostras jovens* Crassostrea corteziensis *e* Crassostrea sikamea *cultivadas artificalmente foi investigado para determinar seu potencial probiótico. Microorganismos foram adicionados à água de cultura a 1×10^6 CFU/ml uma vez por dia durante 30 dias e todas as ostras foram alimentadas diariamente com uma mistura de microalgas. Jovens C. sikamea tratados com culturas N7, RL8 e V4 tiveram ganho de peso significativo quando comparado ao grupo de controle. Jovens* C. corteziensis *tratados com culturas RL8 e BMix demonstraram peso significativamente mais algo que o grupo de controle. Nenhum aumento em tamanho foi observado em grupos tratados em ambas espécies*. *A atividade SOD foi significamente aumentada em C. sikamea treatado com RL8 e com RL8, N7 e BMix em* C. corteziensis. *Culturas* Streptomyces *RL8 e N7 surgem como agentes probióticos promissores para o cultivo de* C. sikamea e C. corteziensis *e podem ser úteis para outros moluscos animais marinhos invertebrados.*

Palavras-chave: actinomyces, ostra, cresimento, efeito probiótico, atividade SOD

INTRODUCTION

Industrial-scale oyster production along the Pacific coast of Mexico began more than 40 years ago with the introduction of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793), which is still the most extensively produced oyster species in this and in other countries worldwide. Other cultivated species are the Kumamoto oyster *Crassostrea sikamea* (Anemiya, 1928), initially introduced to Mexico as a variant to *C. gigas*, as well as the pleasure - oyster *Crassostrea corteziensis* (Hertlein, 1951), a native species found from the Gulf of California to Panama, which is fairly abundant in Mexico's northwestern states of Sonora, Sinaloa and Nayarit states (Mazón-Suástegui *et al.*, 2011).

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Recurrent disease outbreaks with high mortality rates of cultivated oysters are a major problem that seriously reduces mollusc production in many countries around the world (Pulvenis, 2014). These epidemics are commonly produced by mesophilic marine bacteria of the genus *Vibrio*, which affects larval and post-larval stages in seed-producing commercial hatcheries, as well as juveniles and adults grown-out in the field (Travers *et al.*, 2015). For decades, these outbreaks have been treated in hatchery with antibiotics that, over time, increased the pathogenicity of many bacteria, reduced shellfish immune response, and left residual compounds in effluents that spread the problem to more distant locations (Zorriehzahra *et al*., 2016).

In the last few decades, more ecofriendly approaches such as the use of probiotics to control pathogenic bacteria that affect aquaculture have been extensively considered (Tan *et al*., 2016). Probiotics have become a valuable alternative to treat cultivated species because some bacterial strains are able to improve the growth rate, food conversion efficiency, tolerance to stress and immune response of fish and shellfish (Xia *et al.*, 2014). Current scientific knowledge about the use of probiotics is less abundant in bivalve molluscs than in shrimp and marine fish, but growing interest and recent advances in this field are promising, particularly in larval and juvenile stages (Abasolo-Pacheco *et al*., 2016; 2017).

Marine Actinomycetes, mainly of the genus *Streptomyces*, are gram-positive bacteria that may produce antimicrobial secondary metabolites, contribute to the recycling of organic matter and excretion products (Ser *et al*., 2015), and produce heat and desiccation resistant spores (Chater *et al*., 2010). Consequently, Actinomycetes may be an alternative to control disease outbreaks in hatcheries, though very few studies to determine their probiotic effect in marine organisms, including bivalve molluscs, have been conducted (Das *et al*., 2010; García-Bernal *et al*., 2018).

The potential probiotic effect of *Streptomyces* sp. and *Bacillus* mix strains on the growth, survival, and antioxidant activity of superoxide dismutase enzyme is described in juvenile *C. sikamea* and *C. corteziensis* juveniles, two edible oyster species of high commercial value.

MATERIALS AND METHODS

The probiotic strains used in this study were *Streptomyces* strains. N7, RL8, and V4, isolated from marine sediment and selected in previous *in vitro* studies (García-Bernal *et al*., 2015); a *Bacillus* mixture (BMix), composed by *Bacillus tequilensis* YC5-2, *Bacillus endophyticus* C2-2, and *B. endophyticus* YC3-B (Luis-Villaseñor *et al*., 2011), belonging to CIBNOR's culture collection.

All microorganisms were cultivated in tryptone soy broth (TSB; #236950, BD Difco, Franklin Lakes, NJ). *Streptomyces* strains were incubated by shaking at 30°C for 7 days and *Bacillus* strains were incubated at 35°C for 24–48 hours. Cultures were centrifuged at 4696×g for 10min at 4°C, followed by discarding the supernatant and washing the pellet twice with sterile seawater. *Streptomyces* biomass was then re-suspended in sterile seawater until an optical density of 1.0 was reached at 600nm (Gopalakrishnan *et al*., 2014). *Bacillus* biomass was similarly treated until an optical density of 1.0 was reached at 540nm (Luis-Villaseñor *et al*., 20152011). The resulting suspensions 1×10^9 CFU/ml were further adjusted to 1×10^6 CFU/ml for each treatment and were correlated with colony forming unit (CFU) counts using tenfold dilutions and spread plating on tryptone soy agar (TSA).

The experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Mexican Government (NOM-062—ZOO-1999) for the production, care and use of experimental animals, and with the ARRIVE guidelines. Healthy juvenile ostreid molluscs belonging to the species *C. sikamea* and *C. corteziensis* were obtained from Acuacultura Robles Company, Bahía de La Paz, Baja California Sur, Mexico. Animals were acclimatized for 7 days at the Experimental Laboratory of Molluscs of CIBNOR.

Juvenile *C. sikamea* (17±1.20mm shell length; 1.11±0.10g wet weight) and *C. corteziensis* (21±0.65mm shell length; 0.83±0.14g wet weight) were reared in a commercial hatchery. Oysters were kept in 4L plastic containers filled with 1L filtered (1µm) and UV-sterilized seawater, at 29±1°C and 37g/l of salinity with constant aeration. Containers were placed in

large fiber glass tanks operating as recirculating water bath systems to maintain a constant temperature. Containers held 450 oysters divided into 5 treatments of 30 oysters each, with were managed in triplicate. The treatments consisted of (i) *Streptomyces* strain V4; (ii) *Streptomyces* strain RL8; (iii) *Streptomyce*s strain N7; (iv) BMix (1:1:1 ratio) and (v) Control (without probiotics and only fed a microalga mix, as described below). Containers were drained daily, washed and refilled with clean, sterilized seawater. All groups received the microalgae diet first and the bacterial strain 15min thereafter. Treatments were added daily to each container for 30 days.

Two commercial microalgae *Isochrysis galbana* (v. *affgalbana*) and *Chaetoceros calcitrans*, obtained from CIBNOR's collection, were used as food for oysters. Microalgae were cultured in accordance to traditional and standardized methods at CIBNOR laboratory. The diet was provided daily at 1:1 ratio estimated by cell count, and a density of 70-80 $\times 10^3$ cells/ml. At the end of the trial, growth and survival of oysters were recorded for each treatment. With these data, the absolute growth and weight (mm and g, respectively) as well as the survival rate for each treatment was determined at the end of the trial.

Superoxide dismutase (SOD) activity was determined at the end of the experiment (30 days). The soft tissue from six juvenile oysters collected per each of three replicas per experimental group was excised and homogenized (100mg) in 500µl buffered phosphate (pH 7.5), using a tissue homogenizer. The homogenate was centrifuged at 9327×g for 10 min at 4°C. The supernatant was stored at –20°C until used. Superoxide dismutase (SOD) activity was measured using a commercial kit (#19160, Sigma-Aldrich, St. Louis, MO). Results were expressed as an indirect measure of the SOD activity by the percentage reaction inhibition rate of the formation of WST-1 Formazan complex (Dudone *et al*., 2009).

Normality was initially analyzed with the Kolmogorov-Smirnov test and then confirmed with the Levene test for homogeneity of variance (Sokal and Rohlf, 2000). Thereafter, one-way ANOVA was used to assess differences in the growth and SOD activity among experimental groups. The percentage data (SOD) were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P< 0.05$. The analysis was performed using SPSS statistics (v 21 for windows; SPSS Inc., Chicago Il.).

RESULTS AND DISCUSSION

Different responses in weight gain (WG), size increase (SI) and survival rate (SR) of *C. sikamea* and *C. corteziensis* juveniles were attained when treated with three different *Streptomyces* strains and the BMix mixture (Table 1). *C. sikamea* oysters treated with V4, RL8 and N7 had a significant WG, compared to the control group and BMix, ($P< 0.05$). A significant weight gain also occurred in *C.corteziensis* treated with RL8 and BMix ($P< 0.0 5$), but not with V4 and N7 ($P> 0.05$). No significant size increase occurred between probiotic treated groups and the control group ($P> 0.05$) for both oyster species (Table 1). There was no mortality associated to any probiotic treatment during the 30 day trial.

Table 1. Effect of *Streptomyces* strains N7, RL8 and V4 and a *Bacillus* mixture (BMix) on growth parameters of *C. sikamea* and *C. corteziensis* juveniles

Treatments	*C. sikamea*			*C. corteziensis*		
	WG (g)	SI (mm)	SR (%)	WG (g)	SI (mm)	SR (%)
V4	0.22 ± 0.07^{a}	2.14 ± 0.48^{a}	100±0.0	0.10 ± 0.05^{c}	0.13 ± 0.38^{b}	100±0.0
RL8	0.28 ± 0.05^{a}	1.54 ± 0.77^{a}	100±0.0	0.22 ± 0.012^{a}	1.30 ± 0.26^{a}	100±0.0
N7	0.29 ± 0.03^{a}	2.06 ± 0.13^{a}	100±0.0	0.21 ± 0.03^{ab}	1.18 ± 0.13^{a}	100±0.0
BMix	0.07 ± 0.04^{b}	1.31 ± 0.60^{a}	100±0.0	0.23 ± 0.02^{a}	0.33 ± 0.19^{b}	100±0.0
Control	0.06 ± 0.02^{b}	1.21 ± 0.50^{a}	100±0.0	0.07 ± 0.06^{bc}	0.78 ± 0.07^{ab}	100±0.0

Note: Size Increase (SI), Weight Gain (WG) and Survival rate (SR). Data are expressed as mean ± standard deviation. Means in the same column with different superscripts are significantly different ($P< 0.05$).

Probiotics have been associated with either weight increase or decrease in humans and animals (Angelakis *et al*., 2013). The efficacy of probiotics might be species dependent (Mohapatra *et al*., 2012) and dose dependent (Vine *et al*., 2006). In this study, *Streptomyces* strain RL8 significantly increased the weight of juvenile *C. sikamea* and *C. corteziensis* on the control group; this could be due to the ability of this genus to produce extracellular enzymes such as proteases, lipases and amylases (Barka *et al.*, 2016). García-Bernal *et al*. (2015) showed that *Streptomyces* spp. RL8 and N7 produce hydrolytic enzymes that can improve the amylolytic and proteolytic activities in the digestive tract of aquatic organisms. Campa-Cordova *et al*. (2009) reported a growth increase in *C. corteziensis* juveniles with a daily dose of 5×10^4 CFU/ml of *Lactobacillus* sp. strain NS6.1, isolated from the lion-paw scallop *Nodipecten subnodosus*. The probiotic *Lactobacillus acidophilus* increased the weight and survival of pearl oyster, *Pinctada margaritifera*, spat (Subhash and Lipton, 2007), whereas *Pseudoalteromonas* strain X153 reduced the weight of the king scallop *Paralongidorus maximus* Longeo (Longeo *et al*., 2004). Aguilar-Macias *et al*. (2010) reported that hatchery-reared *Pinctada mazatlanica* juveniles significantly increased growth and survival when provided daily with a *Lactobacillus* strain isolated from *N. subnodosus* at 1×10^6CFU/ml. According to Granados-Amores *et al*. (2012), *Pseudomonas aeruginosa* in combination with *Burkholderia cepacia* also beneficially influenced the growth and survival of *N. subnodosus* juveniles.

SOD activity in *C.sikamea* was only significantly higher ($P< 0.05$) with the strain RL8, compared to the control group (Figure 1A). Strains N7, RL8 and BMix induced a significant increase ($P< 0.05$) of SOD activity in *C. corteziensis*, in contrast to the strain V4 which significantly decreased SOD activity in this species (Figure 1 B).

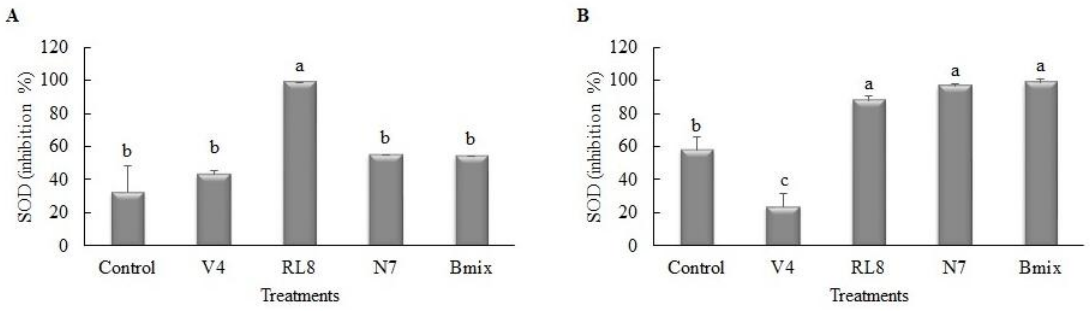

Figure 1. Superoxide dismutase (SOD) activity in (A) *Crassostrea sikamea* and (B) *Crassostrea corteziensis* juveniles after 30 day treatment with *Streptomyces* strains and a *Bacillus* mixture (BMix). Data are expressed as mean ± standard deviation. Bars with different letters are significantly different ($P< 0.05$) from the control group.

Marine invertebrates, including mollusks, have developed an innate immune system, which mainly relies on phagocytosis and the generation of antimicrobial peptides and reactive oxygen species (Lemaitre and Hoffmann 2007). Because reactive oxygen species (ROS) (Dunone *et al*., 2009), mostly superoxide anions, also damage host tissues, invertebrates counteract their effect by means of the enzymes catalase, glutathione peroxidase and SOD (Fridovich, 1998). SOD facilitates the dismutation of superoxide radicals to form hydrogen peroxide, which is subsequently removed by catalase and glutathione peroxidase (Warner, 1994). SOD activity not only protects organism from superoxide anions but also from hydroxyl radicals, which are the most oxidant ROS (González and Arenas, 2002). Thus, tissue and hemolymph SOD activity can be used to measure the immune system competence and the immunomodulatory effect of bioactive substances and probiotics in mollusks (Campa-Córdova *et al*., 2009).

A number of studies have suggested that some probiotics are effective in improving the non-

specific immunity of aquaculture animals (Wang *et al*., 2008). The present study also confirmed these findings. In this study, maximum SOD activity was induced by the strain RL8 with significantly higher values in both oyster species, followed by N7 and BMix, which had greater activity in *C. corteziensis*. Thus, these strains may be used to protect animals from reactive oxygen species generated by infectious diseases or stress during intensive farming. Abasolo-Pacheco *et al.* (2017) showed that the action mechanism of probiotic strains is stage and strain specific, generating different responses by the host, including improved survival and growth (likely from better nutrient assimilation) and higher resistance against pathogens (possibly from strengthening the immune system.

Actinomycetes produce a wide array of antibiotics (Barka *et al*., 2016), which make them promising probiotic candidates in aquaculture. Yet, these microorganisms have been overlooked for this purpose, and very few studies have shown that the genus *Streptomyces* increased growth, survival, and resistance to disease in the giant tiger prawn *Penaeus monodon* (Das *et al*., 2010) and in the *Litopenaus vannamei* (García-Bernal *et al.*, 2018). In our study, *Streptomyces* RL8 was the best performing strain among the other probiotics, as it significantly increased the weight and SOD activity of the two Ostreidae species of commercial and aquaculture interest; closely followed by *Streptomyces* N7 that increased the weight of *C. sikamea* and the SOD activity of *C. corteziensis*, as well as BMix that increased the weight and SOD of *C. corteziensis*.

CONCLUSIONS

Our study shows that the *Streptomyces* strains exert an overall probiotic effect on oysters by improving growth parameters and SOD activity. In addition, this is the first study showing the probiotic effect of actinomycetes strains in juvenile stages of two commercially valuable marine bivalves.

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