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Reducing stress by improving performance of hatchery-reared Catarina scallop (*Argopecten ventricosus*) spat with different genera of beneficial microorganisms: A biochemical and molecular analysis

Ma. del Rocío Vega de la Vega[a], Pedro E. Saucedo[b], Rosa M. Morelos-Castro[b], Roberto Hernández[b], Carlos J. Cáceres-Martínez[a], José M. Mazón-Suástegui[b], Edilmar Cortés-Jacinto[b], Ángel I. Campa-Córdova[b,*]

[a] Universidad Autónoma de Baja California Sur (UABCS), La Paz, B.C.S., Mexico
[b] Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, B.C.S., Mexico

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ABSTRACT

This study investigated the probiotic effects of one lactobacillus, one marine yeast, mix of three bacilli and mix of all probiotic strains on the growth, survival, antioxidant response (superoxide dismutase = SOD, catalase = CAT, gluthatione peroxidase = GPX) and immune response (lysozyme = LYZ, heat shock protein = HSP70) of hatchery-reared Catarina scallop *Argopecten ventricosus* early spat. The spat was exposed every three days for 28 days to a single dose of 1×10^6 CFU mL^{-1} of one of five treatments used in triplicate: (1) *Lactobacillus plantarum* (C3); (2) *Candida cretensis* (S10); (3) Mix of *Bacillus cereus, Bacillus flexus,* and *Bacillus firmus* (BMix); (4) Mix of 1 + 2+3 (LLBMix); (5) Group of spat without probiotics as control. Survival was 100 % in all probiotic treatments and the control group. The spat treated with the yeast (S10) and lactobacillus (C3) significantly increased absolute growth and growth rate compared to the control. The antioxidant activity and gene expression was significantly lower in all spat treated with probiotics than in the control group, particularly with the BMix, LLBMix (SOD), C3 (CAT, GPX), and BMix (CAT) strains. The immune activity and gene expression was also significantly lower in spat treated with probiotics, particularly BMix, LLBMix (LYZ), C3, BMix, and S10 (HSP70) strains, compared to the control group. The use of a single dose of probiotic or mix of different strains reduced activation of antioxidant and immune response and improved overall performance of hatchery-reared *A. ventricosus* spat.

1. Introduction

The Catarina scallop *Argopecten ventricosus* (Sowerby, 1853) is one of the pectinids of greatest commercial value and highest rearing potential in northwest Mexico, given its fast growth rate and soft texture and organoleptic properties of its adductor muscle (Soria et al., 2013). For decades, production of this species relied on artisanal fishing, but this activity was always erratic and inconsistent with productions ranging from 2500 tons in 1998 to 15,800 tons in 2008 (Maeda-Martínez, 2002; FAO, 2019). Thus, hatchery-rearing of *A. ventricosus* larvae and post-larvae (spat) emerged as a potential strategy for supporting fisheries and sustaining aquaculture programs for gradual improvement of wild populations (Corpuz et al., 2014). However, maintaining and controlling rearing conditions at the hatchery has been difficult, due to the use of seawater of varying quality and proliferation of new strains of pathogenic bacteria causing massive high die-offs of larvae and spat and considerable economic losses to the industry (Abasolo-Pacheco et al., 2017). In addition, setting static conditions at the hatchery may impose stressful conditions to developing larvae and spat, affecting their antioxidant and immune response, as well as their susceptibility to some bacterial diseases (Wang et al., 2012).

In marine bivalves, oxidative stress under hatchery conditions may be temporal (acute) or permanent (chronic) and trigger a battery of physiological problems, including slow growth, reproductive failure, loss of homeostasis, increase in reactive oxygen species (ROS), cellular damage, and even death (Lu et al., 2015; Yue-Xin et al., 2019). Oxidative stress may also affect other biological processes of developing larvae and spat at the hatchery, such as metamorphosis and settlement

* Corresponding author at: Centro de Investigaciones Biológicas del Noroeste (CIBNOR). Av. Instituto Politecnico Nacional 195, Col. Playa Palo de Santa Rita Sur, La Paz, Baja California Sur 23096, Mexico.
E-mail address: angcamp04@cibnor.mx (Á.I. Campa-Córdova).

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(Abasolo-Pacheco et al., 2017). Therefore, understanding and controlling the causes and consequences of oxidative stress is essential to improve growth and performance of the species and reduce economic losses to the industry (Lushchak, 2011). In mollusks, the control of oxidative stress is regulated by different types of agents from innate antioxidant and immune system (Mydlarz et al., 2006; Mendoza-Maldonado et al., 2018), including chaperones or heat shock proteins (Yang et al., 2011), enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) (Woo et al., 2013) and surrounding hemocytes (Yang et al., 2017). However, the knowledge related to oxidative stress control appears to be species-specific and is unavailable for many bivalve species, including *A. ventricosus*.

During the last decades, probiotics have proved to be a reliable tool for improving the health of many reared species, by modulating their digestive, antioxidant, and immune responses (Tovar-Ramírez et al., 2010; Li et al., 2019). With mollusks, relatively few studies have analyzed the effects of different bacterial strains on the survival, growth, and antioxidant activity of larvae and spat exposed to pathogens in species such as *Crassostrea corteziensis* (Campa-Córdova et al., 2009), *Nodipecten subnodosus* (Granados-Amores et al., 2012), *Pecten maximus* (Genard et al., 2014), and *A. ventricosus* (Abasolo-Pacheco et al., 2016). In general, most of the knowledge relative to innate immunity of bivalves treated with probiotics is based on functional assays, and almost nothing is known about the molecular basis. A reliable strategy for overcoming this problem is analyzing gene expression under a particular stressor to identify the genes triggering the defense mechanisms (Woo et al., 2013).

This study used a biochemical-molecular approach to identify the enzymes and genes associated with survival, growth, and particularly antioxidant/immune response of hatchery-reared Catarina scallop early spat treated with different probiotic strains. This is a strategy to identify biomarkers of physiological stress that, not only improve performance of larvae and spat at the hatchery and the wild, but reduce economic costs to the industry.

2. Material and methods

2.1. Origin of spat

A. ventricosus spat originated from ripe broodstock collected at the wild in Bahia Magdalena, Baja California Sur, Mexico (24°35′N 112°00′W) and immediately induced to spawn at the hatchery for rearing larvae until the settlement stage.

2.2. Culture of probiotic strains

Microbial strains were obtained from the collection of Centro de Investigaciones Biológicas del Noroeste (CIBNOR), which included one mix of three bacilli (*Bacillus cereus*, *Bacillus flexus*, *Bacillus firmus*) isolated from the digestive tract of lion's paw scallop *Nodipecten subnodosus*, one lactobacillus (*Lactobacillus plantarum*) isolated from the digestive tract of the winged pearl oyster *Pteria sterna*, and a marine yeast (*Candida cretensis*) isolated from mangrove sediment. The bacterial strains were selected from previous *in vitro* trials for hemolytic activity, antagonism against *Vibrio* spp., bacterial hydrophobicity, and production of extracellular enzymes, as well as *in vivo* trials testing and growth of juvenile Kumamoto oyster *Crassostrea sikamea* (Abasolo-Pacheco et al., 2016). The marine yeast *C. cretensis* was selected for its immune-stimulation and bioremediation capacity during rearing of juvenile white-legged shrimp *Litopenaeus vannamei* (Ibarra-Serrano, 2018).

Microbial strains were cultured in Erlenmeyer flasks (100 mL) with Man, Rogosa, Sharpe growth medium (MRS; #288210, Bioxon BD Difco, Franklin Lakes, NJ) for *Lactobacillus* spp., in Tryptic Soy Agar medium (TSA; #210800, Bioxon BD Difco) for *Bacillus* spp., and in Yeast Peptone-Dextrose Broth medium (YPD, Cat #Y1375; Sigma-Aldrich, St Louis, MO) for *C. cretensis*. All strains were incubated for 24 h at 30 °C (Andlid et al., 1995). The cells were separated by centrifugation (4696 *g*, 4 °C for 10 min), re-suspended in sterile seawater, and adjusted to an optic density of 1.0 (540 nm) for bacteria (Harzevili et al., 1998) and 1.0 (620 nm) for the yeast until a concentration of 1×10^9 CFU mL^{-1} was obtained. This density was further adjusted to a final concentration of 1×10^6 CFU mL^{-1} for all treatments (Abasolo-Pacheco et al., 2017).

2.3. Experimental design

A. ventricosus spat (4.5 ± 0.6 mm shell height) were transferred to experimental units consisting of 4 L plastic containers with 2 L filtered (5 µm) and sterilized (UV) seawater at 23 ± 1 °C and 36 PSU. Each unit (used in triplicate) held 100 spat exposed to one of four treatments with probiotics: (1) *C. cretensis* (S10); (2) *L. plantarum* (C3); (3) Mix of *B. cereus, B. flexus, B. firmus* (BMix; 1:1:1 ratio); and (4) Mix of S10, C3, and B-MIX (LLBMix; 1:1:1 ratio). A fifth treatment of spat with no probiotics and fed only with a 1:1 blend of the microalgae *Tisochrysis lutea* and *Chaetoceros calcitrans* (1:1 ratio; 50×10^3 cel mL^{-1}) strains was included as the control group. Daily as a routine for all treatments, the spat received the microalgae first and the probiotics 15 min later, which were directly added to the culturing seawater. Containers were drained, washed, and refilled with clean, sterilized seawater every 48 h. The trial lasted 28 days.

2.4. Survival and growth of spat

For each treatment and replicate, registers for survival (%), absolute growth in shell height (mm), and growth rate (mm d^{-1}) of spat were taken every seven days for 28 days. At each sampling, live spat was counted to estimate mean survival. They were then photographed and processed with Image Pro Plus (v. 9.0, Media Cybernetics, Bethesda, MD) to determine increase in shell size. Finally, the growth rate was determined with the difference between initial and final sizes of spat in relation to experimental time.

During the final biometry, samples of three spat from each replicated treatment (n = 9) were randomly collected to excise and preserve the visceral mass at −80 °C for enzymatic analyses. A similar spat sample was collected and preserved in 1.5 mL Eppendorf tubes with RNA*later* (Sigma-Aldrich, Dorset, UK) for gene expression analyses.

2.5. Enzymatic analysis

Preserved tissues were homogenized in cold saline solution with phosphate buffer (PBS; 0.05 M; pH 6.2), at 20 % w/v proportion (1 g tissue in 8 mL PBS). After centrifuging the homogenates (5000 g; 10 min), the recovered supernatants were used for determining the concentration of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and lysozyme (LYZ). As a previous step, protein concentration was determined with the bicinchoninic acid assay of Smith et al. (1985). SOD activity was determined with a commercial kit (SOD Assay Kit-WST; Cat #19160, Sigma-Aldrich, MO), following the manufacturer's instructions. The protocol of Johansson and Borg (1988) was used for determining CAT activity, based on the reaction of the enzyme with methanol in presence of H_2O_2 Sigma-Aldrich; #707,002(Sigma-Aldrich; Cat #707,002). For determining GPX activity, the method of Lawrence and Burk (1976) was adopted to quantify the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH). LYZ activity was estimated as recommended by Kim and Austin (2006) to measure the cellular wall lysis of *Micrococcus luteus* (Sigma-Aldrich). All readings were taken in triplicate in a microplate reader (Bioteck Instruments Inc., Winooski, VT). The activities of all enzymes were reported as U (mg) protein^{-1}, where one unit represents the minimum change in absorption of 1 mg protein.

Table 1
Primer sequences used for quantitative real-time PCR (qPCR).

Gen	Primer Sequence 5′-3′	Product size (bp)	Annealing temperatura (°C)	Amplification efficiency (%)
Ubiquinone (*CoQ10*)	F-CCATTTTTGGGTTGTTGAGG R-ACCAGCATCAAAGGAACCAG	93	60	106.1
Ribosomal protein L8 (*rpL8*)	F-CGTCATGGATACATCAAGGGT R-CAAACAGTCCAGTGTACATGCC	154	60	91.4
Alpha tubulin 1a (*tub-α 1a*)	F-GCACAGTCGGAGTGCTCAAG R-GGTGGWACCGGATCWGGATTCA	176	60	91.3
Ribosomal protein 60S (*60S*)	F-AGGTATCTGGTCATGCAAACG R-TCTTACTGTAGCGGCAGCATT	85	60	97.5
Ribosomal RNA 18S (*18S*)	F-GAAATTCTTGGATCGCCGTA R-GCCGAGTCATTGAAGCAACT	168	60	99.7
Lysozyme (*lis-g*)	F-CAATGGATGGGGAGACCA R-GCCACCTGATTGATGTAGGG	147	60	121.5
Heat Shock protein (*hsp70*)	F- GCGTAACACAACTGTCCCCAC R- TCATTGCTCGTTCTCCCTCG	104	60	109.0
Gluthatione peroxidase (*gpx*)	F-ATGGAGACATGGGCTTTCAG R-GTTCGTTCTTGCCGTTGATT	174	60	100.4
SuperoxideDismutase (*mnsod*)	F-AATAGGGATTTTGGCTCGTTTG R-TGGTTGAAGTGGGTCCTGGTTA	153	60	96.7

2.6. Gene expression analysis

2.6.1. Isolation of RNA and synthesis of cDNA

Preserved samples were rinsed in nuclease-free water and homogenized in 800 µL TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN). RNA was isolated by chloroform extraction, ethanol precipitation, and 70 % ethanol washes. Pellets were resuspended in 30 µL nuclease-free water. After determining RNA quantity and quality with Nanodrop-1000 (ThermoScientific, Chicago, IL), its integrity was verified in 1% agarose gel with 1X TBE buffer (Tris-Boric acid-EDTA) with 1x GelGreen (Biotium Inc., Fremont, CA) and visualized in an UV Transilluminator (UVP Inc., Upland, CA). Total RNA was treated with RQ1 RNAse-free DNAse (Promega, Madison, WI) according to the manufacturer's instructions. Absence of genomic DNA contamination was confirmed by PCR non-amplification using riboprotein L8 (rpL8, Table 1) oligonucleotides (0.24 µM each), 0.125 U GoTaq Flexi DNA Polymerase (Promega), 2.5 mM $MgCl_2$, 1x Go Taq Flexi Buffer, 0.25 mM dNTP Mix (Promega), and 1 µl RNA-treatment, in a final volume of 12 µL Vandesompele et al. (2002). The PCR was performed in a thermocycler (T-1000, Bio-Rad Laboratories, Hercules, CA) using the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and a final extension at 72 °C for 10 min. PCR non-products were visualized on a tris-borate-EDTA (TBE) with 1% agarose gel as described before. A total of 2 µg DNA-free RNA was used for complementary DNA (cDNA) synthesis, using Improm-II reverse transcription system (Promega), mix of oligo (dT) 15 primer (Sigma-Aldrich) with Random Primers (Promega), and RNAsin Plus RNAse inhibitor (Promega) in a volume of 20 µL. The resulting cDNA was stored at −20 °C.

2.6.2. Expression of genes related to the immune system

Specific primers for the expression of the manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPX), lysozyme (LYZg), heat shock protein (HSP70), and two reference genes including *ubiquinone* (CoQ10) and *60 s* (ribosomal protein 60 s) were designed for *A. ventricosus* (Table 1). Reference gene primers from *rpl8* (riboprotein l8), *tub-α* (alpha-tubulin), and *18S* (18S-ARNr) were designed from a sequence alignment of different mollusks. The immune system primers (target genes) and reference genes used for Real time PCR analyses are shown in Table 1.

Real time PCR was performed with 5 µL cDNA (1:50 dilution), using a mixture of EvaGreen 2 × PCR, as recommended by Llera-Herrera et al. (2012). Amplification conditions were: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s (to acquire fluorescence) and 72 °C for 15 s. After each reaction, a dissociation curve (60–95 °C) was constructed, with 0.5 °C increments every 5 s. Each individual sample was measured in triplicate and a negative control was added. PCR amplification was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) with 96-well clear low-profile PCR microplates and ultraclear sealing film (Bio-Rad). Standard curves were calculated for each gene to determine efficiency (E) (Bustin et al., 2009) using six serial dilutions (factor 1:5) in triplicate of the cDNA pool samples. A stability analysis was performed with five reference genes: *rpl8*, *tub-α*, *ubiquinone*, *60S*, and *18S*. Cq values for all samples were evaluated using four computational algorithms (ΔCt, NormFinder, BestKeeper, and GeNorm) integrated into the RefFinder software package (Xie et al., 2012). The relative expression (RE) of target genes was normalized with the reference gene *ubiquinone*. The RE of each target gene was calculated with the equation $RE = RQ_t / RQ_{nf}$, where *t* is the target gene and *nf* is the normalization factor, as mentioned in Hellemans et al. (2007).

2.7. Statistical analysis

Group normality was initially analyzed with the Kolmogorov–Smirnov test and then confirmed with the Levene test for homogeneity of variances. Thereafter, one-way ANOVA was used to assess for significant differences in growth, enzymatic/immune response, and expression of genes between probiotic treatments. As needed, *post hoc* multiple range mean comparisons with Tukey's test (HSD) were included. The level of significance was set at $P < 0.05$ for all analyses. The SPSS software (V. 24.0, SPSS Inc., IBM Co., Armonk, NY) was used.

3. Results

3.1. Survival and growth

After 28 days rearing, the spat treated with the marine yeast (S10) and lactobacillus (C3) grew significantly ($P < 0.05$) larger and faster in shell height than the control spat (Table 2). Spat exposed to mixed microorganisms (BMix and LLBMix) did not show significant ($P > 0.05$) differences in absolute growth and growth rate compared to the

Table 2
Absolute growth and growth rate of *Argopectenventricosus* spatexposed to probiotics.Mean ± correspond to standard error (SE). (*) Significantly ($P < 0.05$) different than control.

Treatment	Absolute Growth (mm)	Growth Rate (mm day^{-1})
Control	8.6 ± 0.1	0.15 ± 0.04
S10	9.6 ± 0.2 *	0.19 ± 0.06 *
C3	9.3 ± 0.1 *	0.18 ± 0.04 *
BMix	9.1 ± 0.1	0.17 ± 0.04
LLBMix	9.0 ± 0.1	0.17 ± 0.05

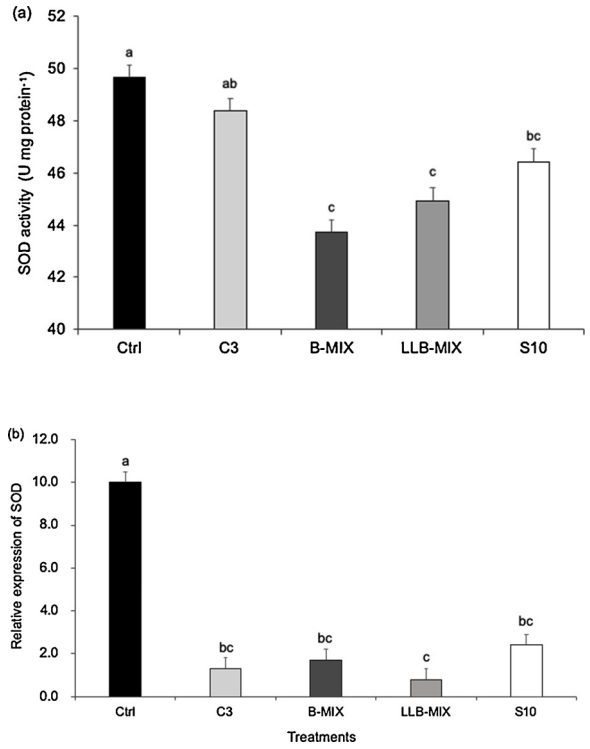

Fig. 1. Superoxide dismutase (SOD) activity (a) and relative expression of SOD (b) in *Argopecten ventricosus* spat treated with probiotic strains: (CTRL) Control; (C3) *Lactobacillus plantarum*; (MIX-B) *Bacillus cereus, B. flexus*, *B. firmus*; (MIX-LLB) MIX-B + S10 + C3; (S10) *Candida maris*. Mean ± standard errors are shown. Different superscript letters denote significant differences between treatments ($P < 0.05$).

control group.

3.2. Antioxidant response and its relation to gene expression

3.2.1. Superoxide dismutase (SOD)

The spat treated with the BMix, LLBMix, and S10 strains showed a significant ($P < 0.05$) decrease in SOD activity, compared to the control group where the activity was maximal (Fig. 1a). In contrast, SOD activity did not significantly ($P > 0.05$) vary in spat exposed to the C3 treatment in relation to the control group.

All probiotic strains, particularly LLBMix, promoted a significant ($P < 0.05$) decrease in the expression of the MnSOD gene, compared with the control group (Fig. 1b).

3.2.2. Catalase (CAT)

The activity of CAT was significantly ($P < 0.05$) lower in spat treated with the C3, BMix, and LLBMix strains, and peaked in the spat from the control group (Fig. 2). CAT activity in spat treated with *C. cretensis* was not significantly ($P > 0.05$) different from that of the control group.

3.2.3. Glutathione peroxidase (GPX)

The GPX activity in spat supplemented with *L. plantarum* (C3) and mix of bacilli (BMix) was significantly ($P < 0.05$) lower than in the control group (Fig. 3a). Conversely, the spat exposed to LLB-Mix and S10 did not show significant ($P > 0.05$) differences in the activity of this enzyme, compared to the control.

The expression of the GPX gene significantly ($P < 0.05$) decreased in the spat treated with all probiotic strains and increased in the control spat (Fig. 3b). Particularly, the expression of this gene was the lowest in spat supplemented with *L. plantarum*.

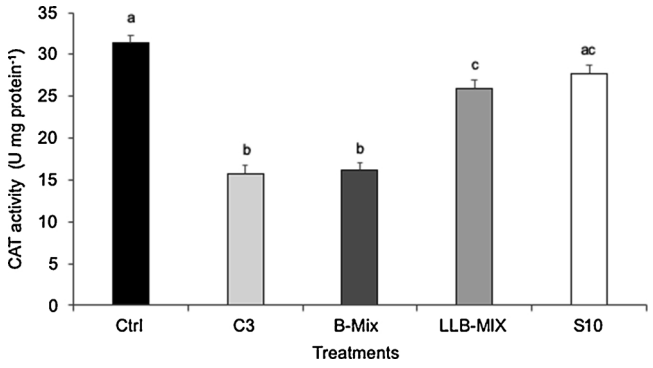

Fig. 2. Catalase (CAT) activity in *Argopecten ventricosus* spat treated with probiotic strains: (CTRL) Control; (C3) *Lactobacillus plantarum*; (MIX-B) *Bacillus cereus, B. flexus*, *B. firmus*; (MIX-LLB) MIX-B + S10 + C3; (S10) *Candida maris*. Mean ± standard errors are shown. Different superscript letters denote significant differences between treatments ($P < 0.05$).

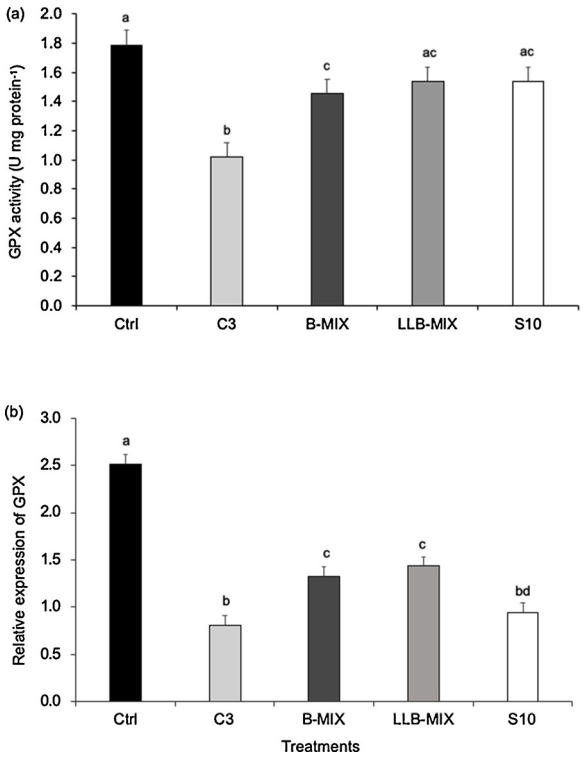

Fig. 3. Glutathione peroxidase (GPX) activity (a) and relative expression of GPX (b) in *Argopecten ventricosus* spat treated with probiotic strains: (CTRL) Control; (C3) *Lactobacillus plantarum*; (MIX-B) *Bacillus cereus, B. flexus*, *B. firmus*; (MIX-LLB) MIX-B + S10 + C3; (S10) *Candida maris*. Mean ± standard errors are shown. Different superscript letters denote significant differences between treatments ($P < 0.05$).

3.3. Immune response and its relationship to gene expression

3.3.1. Lysozyme (LYZ)

The spat supplemented with probiotics, particularly the LLBMix treatment, showed a significant ($P < 0.05$) decrease in the activity of the LYZ enzyme, compared to the control group (Fig. 4a).

A significant ($P < 0.05$) decrease in the expression of the lysozyme gene (LYZg) occurred in all the spat treated with probiotics, particularly with the BMix strain; the expression of this gene was higher in the control spat (Fig. 4b).

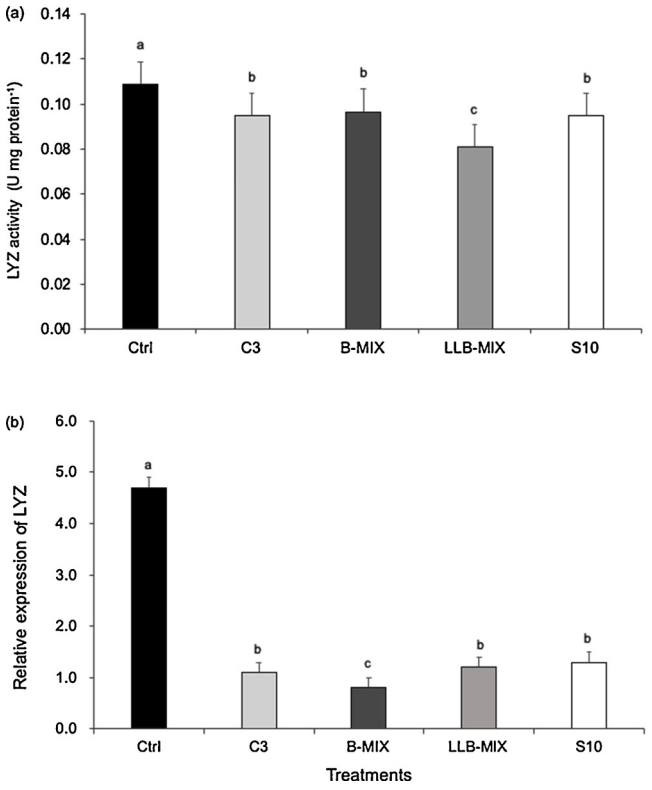

Fig. 4. Lysozyme (LYZ) activity (a) and relative expression of lysozyme (LYZg) (b) in *Argopecten ventricosus* spat treated with probiotic strains: (CTRL) Control; (C3) *Lactobacillus plantarum*; (MIX-B) *Bacillus cereus, B. flexus*, *B. firmus*; (MIX-LLB) MIX-B + S10 + C3; (S10) *Candida maris*. Mean ± standard errors are shown. Different superscript letters denote significant differences between treatments ($P < 0.05$).

3.3.2. Heat shock protein (HSP70)

The relative expression of the HSP70 gene followed the same pattern than the other genes, where a significant ($P < 0.05$) decrease in its expression occurred in all spat treated with probiotics, compared to the control group (Fig. 5). The pattern was particularly lower with the C3, BMix, and S10 strains.

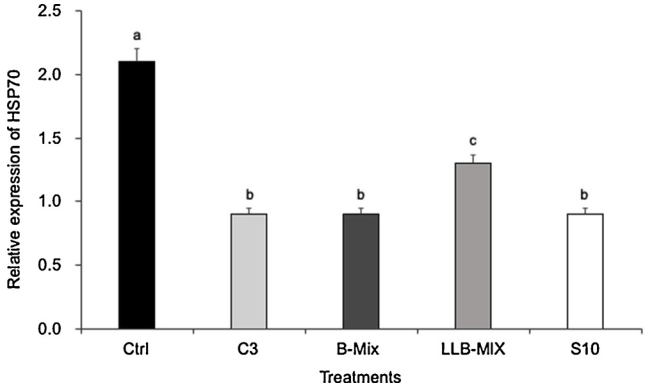

Fig. 5. Relative expression of heat shock protein 70 (HSP70) in *Argopecten ventricosus* spat treated with probiotic strains: (CTRL) Control; (C3) *Lactobacillus plantarum*; (MIX-B) *Bacillus cereus, B. flexus*, *B. firmus*; (MIX-LLB) MIX-B + S10 + C3; (S10) *Candida maris*. Mean ± standard errors are shown. Different superscript letters denote significant differences between treatments ($P < 0.05$).

4. Discussion

Clearly, *A. ventricosus* spat grew significantly larger and faster when treated with the marine yeast *C. cretensis* and lactobacillus *L. plantarum*; in all cases, the poorest growth occurred in the control group. Relatively few studies have analyzed the effects of marine yeasts on bivalve larvae and spat. In the abalone *Haliotis midae*, for example, the yeast strains SSI and AY1 isolated from the digestive tract of the abalone *Haliotis midae* significantly improved its survival, growth and phagocytic activity during a challenge with the pathogen *Vibrio anguillarum* (Macey and Coyne, 2005). In aquaculture, the marine yeast *Candida* spp. have been reported a good food supplement to enhance growth, food intake, and resistance against pathogens in other species of commercial value, such as fish and shrimp. This because of their high nutritional value (Pouluse, 2013), and capacity to stimulate the host's immune response (Abdel-Tawwab et al., 2008; Andrews et al., 2011; Caruffo et al., 2016). This is the case of *Candida sake* and *Candida tropicalis*, which are easy to produce and supply high-quality nutrients that improve the immune system of the Indian white prawn *Feneropenaeus indicus* (Sarlin and Philip, 2011). More studies testing different marine yeast species (alive or processed) are necessary to broaden the understanding of their action mechanisms and physiological response of *A. ventricosus* across all major developmental stages (embryos, veliger larvae, umbonate larvae, pediveliger larvae, and spat).

The lactobacillus *L. plantarum* also favored a significant increase in shell height in comparison with the control group. In general, most acid-lactic bacteria (*e.g. Lactobacillus bulgaricus*, *Lactobacillus acidophillus, Lactobacillus casei, Lactobacillus sporogenes, Lactobacillus plantarum*, and *Streptococcus thermophillus*) have proved to be effective probiotics in aquaculture (Jacobsen et al., 1999; Venkat et al., 2004). Abasolo-Pacheco et al. (2017) observed a significant increase in size and weight of *A. ventricosus* juveniles supplemented with *L. plantarum*. The benefits of this lactobacillus species as growth promotor has also been reported in other marine species, including Nile tilapia *Oreochromis niloticus* (Jatobá et al., 2011), turbot *Scophthalmus maximus* (Gatesoupe, 1991), Indian white shrimp *Penaeus indicus* (Uma et al., 1999), and whiteleg shrimp *Litopenaeus vannamei* (Kongnum and Hongpattarakere, 2012). While the dose used in this study (1×10^6 CFU mL^{-1}) was sufficient to stimulate growth, evaluating new strains and doses of lactobacilli are recommended with larvae and spat to confirm these results and broaden the understanding of the potential use of this bacteria species as immunostimulant at a larger commercial scale. So far, the evidence collected in this study suggests that the effect of *L. plantarum* is dose-specific and stage-specific, similarly to the pattern observed with the yeast *C. cretensis*.

In aquaculture, the genus *Bacillus* spp. has been successfully used to improve seawater quality, reduce the load of harmful bacteria, and maximize the host's response without antibiotics, given its ability to sporulate, grow fast, and tolerate a wide range of physiological conditions (Nemutanzhela et al., 2014). Moreover, using mixes of different bacilli strains instead of monospecific doses offer advantages for enhancing overall health of the host, likely in response to the specific synergy of the mix (Ouwehand et al., 2000; Timmerman et al., 2004; Zhao et al., 2018). In our study, the BMix strain (*B. cereus*, *B. flexus*, *B. firmus*) did not promote a significant increase in shell height of spat compared to the control group, yet it favored a significant reduction in the activity (and expression of genes) of the SOD and CAT enzymes. Recent findings with the same BMix highlight its poor value for *A. ventricosus* larvae that experienced high die-off likely in response to inadequate doses being too high during early developing stages (Abasolo-Pacheco et al., 2017). In contrast, the BMix significantly improved survival and growth of *C. sikamea* spat in comparison to the control spat (Abasolo-Pacheco et al., 2016). Similarly, growth, survival, and pepsin, amylase and/or cellulase activities in umbo larvae, pediveliger larvae, and spat of the Yesso scallop *Patinopecten yessoensis* supplemented with *Bacillus aquimaris* (T16 strain) at low and high

concentrations (1×10^4 and 1×10^6 cells mL^{-1}, respectively) were significantly higher than those in the control group (Yue-Xin et al., 2019). A mix of *B. subtilis* and *B. cereus* also favored a significant improvement in survival and growth in size and weight in the sea cucumber *Apostichopus japonicus* (Li et al., 2015), while a mix of *B. subtilis*, *B. firmus*, and *B. flexus* increased weight in the whiteleg shrimp *L. vannamei* (Setyati et al., 2014). Future studies analyzing the effects of different bacilli strains (alone or combined with other probiotic bacteria), and at different doses, on the antioxidant and immune response of *A. ventricosus* larvae and spat are needed to confirm the potential benefit of this genus in aquaculture. So far, the evidence indicates that the nutritional value and grade of assimilation and digestibility of *Bacillus* spp. as probiotic are species-specific (Brown et al., 1996; Lara-Flores et al., 2003; Tovar-Ramírez et al., 2010).

Overall, the treatment with probiotics significantly reduced the antioxidant response of spat, in comparison to the control group. This was particularly evident with the lactobacillus (C3) and mix of bacilli (BMix). In support to these findings, Granados-Amores et al. (2012) reported a significant decrease in the activity of CAT in spat of the lion's paw scallop *N. subnodosus* treated with the commercial probiotics Epicin® and Bactosafe®. These results are also consistent with the pattern reported for other marine invertebrates and vertebrates experiencing reduction in SOD, CAT, and GPX activities after treatment with probiotics; this is the case of the Pacific blue shrimp *Litopenaeus stylirostris* supplemented with the acid-lactic bacterium *Pediococcus acidilactici* (Castex et al., 2009), larvae of the European bass *Dicentrarchus labrax* fed the CBS 8339 yeast *Debaryomyces hansenii* (Tovar-Ramírez et al., 2010), American yellow perch *Perca flavescens* treated with a commercial mix of *B. subtilis*, *B. pumlis*, *B. amyloliqueficiens*, and *B. licheniformis* (Shaheen et al., 2014), and Orange spotted grouper *Epinephelus coioides* exposed to the lactobacillus *L. plantarum* (Son et al., 2009).

Contrary to the decreasing pattern observed in this study, the activity of antioxidant enzymes has been reported to increase when the species are exposed to certain stressors, such as pollution and heavy metals (Cossu et al., 1997; Canesi et al., 1999), acute or chronic changes in environmental factors (Lesser, 2006; De Almeida et al., 2007), and spread-out of bacterial infections (Cajaraville et al., 2000). For example, Genard et al. (2014) reported an increase in the activities of CAT and SOD enzymes in larvae of the King scallop *Pecten maximus* fed the probiotic bacterium *Phaeobacter gallaeciensis* and then challenged with the pathogen *Vibrio pectenicida*. Abasolo-Pacheco et al. (2017) also noted an increase in the SOD and CAT activities in *A. ventricosus* juveniles fed with probiotics and then exposed to the pathogen *Vibrio alginolyticus*. In contrast, the activity of the LYZ enzyme, and its corresponding LYZ gene, was significantly lower in all the spat given probiotics in our study. Despite of the few available studies dealing with the influence of stress on this enzyme, its activity appears to increase when animals are exposed to acute and chronic stress (Demers and Bayne, 1997), pathogens (Chen et al., 1996), and bacterial infections (Møyner et al., 1993). In bivalves, lysosome has been reported as one of the most important molecules regulating the defense mechanism of many species (Takahashi and Itoh, 2011), and as such, this enzyme may be used as response to stress biomarker (Taoka et al., 2006). Similarly, the expression levels of the HSP70 protein were significantly lower in all probiotic-fed spat than in the control spat. Usually, the expression of chaperone proteins in marine invertebrates is directly tied to their immune system, with values peaking again under conditions where denaturalized proteins need to be repaired (Iwama et al., 1998) or when the number of hemocytes responding to infectious processes is regulated (Cellura et al., 2007; Mello et al., 2012). This is the case of the Atlantic bay scallop *Argopecten irradians*, where the peaking expression of the HSP70 was related to peaking exposure time and dose of the pathogen *Vibrio anguillarum* (Song et al., 2006). Identical responses have been reported for juveniles of other marine species such as the Leopard grouper *Mycteroperca rosacea* (Reyes-Becerril et al., 2011) and gilt-head sea bream *Sparus aurata* (Rollo et al., 2006), which makes the importance of this molecule evident as oxidative stress biomarker.

In summary, the adoption of biochemical-molecular tools to study the effect of probiotic strains provided an efficient approach to enhance survival and growth, and prevent reducing the activation of both antioxidant and immune response in hatchery-reared *A. ventricosus* early spat. The information of this study has potential application to hatcheries to maximize performance, scale production, and reduce economic costs. Further experiments evaluating different stressors (temperature, salinity, water quality, heavy metals) or adverse conditions with developing embryos, larvae, and spat are recommended.

5. Author statement

M. del Rocío Vega De la Vega: Study design and execution; analysis of data; drafting, revision, and final approval of manuscript.

Pedro E. Saucedo: Study design, analysis of data, drafting, revision, and final approval of manuscript.

Rosa M. Morelos-Castro: Analysis of data, drafting and revision of manuscript.

Roberto Hernández: Analysis of data, drafting and revision of manuscript.

Carlos J. Cáceres-Martínez: Drafting and revision of manuscript.

José M. Mazón-Suástegui: Analysis of data; drafting, revision, and final approval of manuscript.

Edilmar Cortés-Jacinto: Analysis of data; drafting and revision of manuscript.

Ángel I. Campa-Córdova: Study design, analysis of data, drafting, revision, and final approval of manuscript.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100298.

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