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Research Article

Effect of immunomodulatory medication over the general response of juvenile Catarina scallop (*Argopecten ventricosus* Sowerby II, 1842)

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ABSTRACT. The future of the bivalve mollusk production sector worldwide is still challenged by emergence and propagation of new diseases, mainly vibriosis, and the conventional methods that have been used to eradicate pathogens from hatcheries, such as antibiotics and chemotherapies have shown collateral effects. In order to reduce the use of conventional methods, homeopathy for aquaculture emerges as a novel solution to strengthen marine organism's defense against diseases. This study evaluated the effect of five homeopathic treatments on the immunomodulatory response and general condition index of *Argopecten ventricosus* juveniles, used as model organisms due to their susceptibility to vibriosis compared to other commercial bivalves. The experimental design included three replicates of five homeopathic treatments at decimal (D) or centesimal (C) dynamization (T1: ViA 1D + ViP 1D, T2: ViA 7C + ViP 7C, T3: AcF 1D + MsS 1D, T4: PhA 7C + SiT 7C and T5: ViT 31C) and three control groups (C1: Dynamized Ethanol (1C), C2: Diluted Ethanol (1:99) and C3: No medication). The maximum immunomodulatory response was attributed to treatments T1 and T2 with the highest increase in hemocyte count, catalase activity in mantle and superoxide dismutase activity in gills, followed by treatments T3 and T5. Treatment T4 did not show a definite effect on an immune response but increased energetic reserves in the mantle, muscle and the digestive gland, important storage tissues in mollusk bivalves. This study has demonstrated that some homeopathic treatments can activate an immunomodulatory response and improve the general condition index in *A. ventricosus*.

Keywords: bivalve; aquacultural homeopathy; immunomodulatory response; superoxide dismutase; catalase

INTRODUCTION

The future of the bivalve mollusk production sector worldwide is still challenged by the emergence and propagation of new diseases that are strongly influenced by water temperature increase due to global warming (Murray *et al*., 2012; Wendling *et al*., 2014; Semenza *et al*., 2017) and organism mobilization (Murray *et al*., 2012). The main limiting factor for hatchery spat production in sea bivalves is the high mortality associated with the disease caused mainly by *Vibrio* spp, and moreover, by *Aeromonas* spp. in early-stage development (Kesarcodi-Watson *et al*., 2008; Sivasankar *et al*., 2017; Stentiford *et al*., 2017). Conventional methods such as antibiotics have been used to combat diseases by attacking pathogenic bacteria, but they negatively affect beneficial gastrointestinal microbiota (Romero *et al*., 2012), and their prolonged use generates antibiotic resistance in more pathogenic bacterial strains (Lawrence & Jeyakumar, 2013). Even governments and organizations in different parts of the world have been restricting the use of antibiotics in animal production (Kesarcodi-Watson *et al*., 2008), but at the same time, there is an increasing need for finding alternative ways to control microbial diseases in aquaculture.

To avoid the massive use of antibiotics, scientists have been searching for sustainable alternatives as immunostimulant (Wang *et al*., 2017) with probiotics (Prado *et al*., 2010; Abasolo-Pacheco *et al*., 2017),

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antimicrobial essential oils (Romero *et al*., 2012), inhibition of virulence genes expression in pathogenic bacteria (Dubert *et al*., 2017) and recently proposed homeopathic drugs (Mazón-Suástegui *et al*., 2017).

Homeopathy, an innocuous and eco-friendly alternative, has been proposed to strengthen biological systems against diseases and uses dilutions from decimal (low-dilution) to millesimal (very high-dilution) potencies (Bellavite & Signorini, 2002). Homeopathy has been considered a pioneer in nano-medicine application for human health because even in high-dilution homeopathic drugs, nanoparticles of the original compound have been found (Bell *et al*., 2013). The action mode of these drugs depends on the potency of the homeopathic drug. Low dilutions effects are attributed to high diluted molecules and nanoparticles, while high dilutions effects are attributed to nanoparticles and electromagnetic fields (Bellavite *et al*., 2014a). These signals or molecules generates endogenous amplification that include hormesis (phenomena where a compound at high concentrations produce a harmful effect and at low concentration, a beneficial effect), time-dependent sensitization and stochastic resonance process (Bell *et al*., 2013; Bellavite *et al*., 2014b). Homeopathic drugs have been previously used in animals, plants, humans and *in vitro* research (Bellavite & Signorini, 2002; Bellavite *et al*., 2014b), showing encouraging results in the activation of the immune response, stress reduction, nutrient assimilation increase and as growth promoters (Bellavite & Signorini, 2002; Bellavite *et al*., 2006; Camerlink *et al*., 2010; Merlini *et al*., 2014; Dias-Neto *et al*., 2017). Most studies have been done in humans, while for aquatic organisms, information is scarce. Nile tilapia *Oreochromis niloticus* has been the most studied aquatic species in the evaluation of homeopathic drugs effect. Researches have been reported changes for *O. niloticus* in lipid metabolism (Andretto *et al*., 2014), stress reduction (Merlini *et al*., 2014), survival (Dias-Neto *et al*., 2017) and growth (Merlini *et al*., 2014) when organisms are treated with homeopathic complexes.

Recently, homeopathic drugs formulated from silica, phosphoric acid, Vidatox® (Havana, Cuba) and pathogenic bacteria have been successfully used in marine organisms to improve and strength their immune systems (Ortiz-Cornejo *et al*., 2017). In mollusks, homeopathic drugs have been used as a novel solution to aid overpass bacterial attack by improving immune response. In Catarina scallop *Argopecten ventricosus*, spat improved survival and increased superoxide dismutase (SOD) activity when challenged against *Vibrio alginolyticus*, a highly pathogenic bacteria (Mazón-Suástegui *et al*., 2017), suggesting that homeopathic drugs have immunological properties and potential protection against vibriosis. Nonetheless, current scientific knowledge of the use of homeopathic drugs in marine bivalves and their implication in the antioxidant system and general condition is scarce.

This study aimed to determine the effect of five homeopathic treatments, that previously shown an immunomodulatory response (Ortiz-Cornejo *et al*., 2017), on growth, antioxidant system, hemocytes count and general condition index in Catarina scallop juveniles under laboratory conditions. This pectinid species was selected as marine bivalve model because it is an important resource in the Baja California Peninsula and highly susceptible to vibriosis (Luna-González *et al*., 2002) which causes massive mortalities at their early-life stage. Therefore, the objective of this study was to assess the potential of homeopathy for aquaculture as a new eco-sustainable alternative to improve hatchery production of spat for aquaculture purposes.

MATERIALS AND METHODS

Origin of scallops and experimental design

A total of 1,500 Catarina scallops (average length 1.98 ± 0.1 cm) were collected from culture cages at La Paz Bay, Mexico (24°9'5"N; 110°20'10"W) in March 2016. Scallops were transported to the mollusks laboratory at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) and maintained in a nursery upwelling recirculating system at 24°C with a food concentration around 150,000 cel mL^{-1} (*Isochrysis galbana* and *Chaetoceros calcitrans* in proportion 1:1) for acclimatization for one week. Filtered (1 µm) and UV-irradiated seawater was continuously provided (24 h), which allows water changed every day.

After acclimatization, scallops were transferred to 24 experimental units (36 L container) with 52 scallops each. During the experimental period (21 days), scallops were kept in an open-recirculating flow system that provided filtered and treated sea water (1 µm, activated carbon and UV irradiation) with a mix of microalgae *I. galbana* and *C. calcitrans* (199,607,794 cel ind^{-1} d^{-1}) at 23.5 ± 0.5°C.

Five experimental homeopathic treatments (T1, T2, T3, T4, and T5) and three control treatments (C1, C2, and C3) were assayed, including three replicates for each experimental condition. To avoid and detect potential side effects of ethanol vehicle in homeopathic treatments, C1 contained diluted (1:100) but not succussed ethanol; C2 diluted 1:100 and succussed ethanol (1C); and C3 neither homeopathic drug or ethanol added. Ethanol is the most typical dilution-

succussion vehicle used in human homeopathic drugs (Bellavite & Signorini, 2002), but it may trigger phenoloxidase activity in shrimps (Hernández-López *et al*., 1996). Distilled water was used as the final dilution vehicle in all "working" (decimal or centesimal) homeopathic dynamizations to avoid this potential effect.

The open flow was cut off three hours a day to avoid the presence of microalgae and permits homeopathic or control treatments were directly added to seawater in the experimental units (100 µl L^{-1}), a dose that had proved to strengthen the immune system in *A. ventricosus* (Mazón-Suástegui *et al*., 2017). During this time, water recirculation pattern in each experimental unit was maintained to promote intake of homeopathic and control substances added to juvenile scallops. Growth and survival were measured every week. At the beginning (T_o, day 0) and the end of the experiment (day 21), 18 scallops were randomly collected from each treatment for analysis purposes (six scallops per unit). For the histological analysis, the whole scallop meat was fixed in Davidson solution. For biochemical and enzymatic analysis muscle, digestive gland, gills, and mantle were excised and maintained at -80°C.

Homeopathic drug formulation

Homeopathic treatments T1 (ViP 1D + ViA 1D) and T2 (ViP 7C + ViA 7C) were homeopathic dynamizations developed at CIBNOR, formulated in decimal (T1) or centesimal (T2) dilutions/succussions from a mother tincture of pathogenic strains of *Vibrio parahaemolyticus* (CAIM 170; www.ciad.mx/caim » ViP) and *Vibrio alginolyticus* (CAIM 57; www.ciad.mx/caim » ViA), which have been related to high mortalities in marine bivalves. For each strain, mother tincture was prepared following the homeopathic principles that consisted of wet biomass obtained by centrifugation (13,000 rpm, 4°C, 20 min) of 2 L of *Vibrio* culture in marine broth 2216 (105.1×10^{-6} CFU mL^{-1}). Wet biomass (15 mL) was fully inactivated by three freeze-unfreeze cycles of -80 and 24°C; between each cycle, strain biomass was vortexed at 3,200 rpm (Benchmark mixer™, Benchmark Scientific Inc.) for 2 min. The inactivated product was diluted (1:1) in ethanol 87% (Similia® purchased at Farmacia Homeopática Nacional®, Mexico City, MX) and vortexed at 3,200 rpm (Benchmark mixer™, Benchmark Scientific Inc.) for 2 min. Concentrate diluted in ethanol was again diluted (1:100) in ethanol 87% (Similia®) and vortexed 2 min at 3,200 rpm (Benchmark mixer™, Benchmark Scientific Inc.) to get a final mother tincture, from which, decimal (D, 1:10) and centesimal (C, 1:100) homeopathic dynamizations were prepared by successive dilution and succussion process (3,200 rpm, 2 min, Benchmark mixer™, Benchmark Scientific Inc.) until reach dynamization 1D (1:10) and 7C ($1:10^{14}$).

Homeopathic treatment T3 (AcF 1D + MsS 1D) was developed from sodium metasilicate and phosphoric acid mother tinctures. Analytic grade phosphoric acid 86% (Faga Lab®, Guamuchil, MX) was considered as mother tincture, while the sodium metasilicate mother tincture was prepared by mixing sodium metasilicate (9.4 g) in distilled water at room temperature (25°C) until the solution reached saturation. Both mother tinctures were succussed and diluted (1:10) to have the dynamization 1D of each formulation.

Homeopathic treatments T4 (PhA 7C + SiT 7C) and T5 (ViD 31C) were elaborated from commercial homeopathic drugs. Treatment T4 consisted of centesimal (C) preparations (1:100 dilution/succussion) of the commercial homeopathic drugs Phosphoricum acid® 6C (PhA, dilution 1×10^{12}) and Silicea terra® 6C (SiT, dilution 1×10^{12}), while treatment T5 (ViD 31C) consisted of a centesimal homeopathic dynamization (1:100 dilution/succussion) of a commercial homeopathic drug (VidatoX® 30CH, dilution 1×10^{60}; Labiofam®, Havana, Cuba) made from the venom of the blue scorpion *Rhopalurus junceus*. Distilled water was used as a final dilution vehicle.

Haemocyte count

The hemocyte count was assessed to prove immune system activation by following the methodology of Bianchi *et al*. (2015). Three selected digital images taken from digestive gland tissue, which showed higher hemocytes proliferation than other tissues, at 100x were processed with Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) to count the number of hemocytes seen in a tissue area (0.21 mm^2).

Antioxidant enzymatic activity

From each sample, previously stored at -80°C, 0.1 g of tissue was homogenized in phosphate buffer 50 mM, pH 7.8 (1:5) to determine enzymatic biochemical activity, using glass beds and a fast prep (2 cycles, 4 m s^{-1}, 30 s) equipment. Samples were centrifuged (13,000 rpm, 4°C, 10 min) and the supernatant was recovered to obtain the final enzymatic extract.

Superoxide dismutase (SOD) and catalase (CAT) activity were measured to understand the effect of homeopathic treatments on the antioxidant system of the Catarina scallop. The SOD activity was determined using the SOD determination kit (Sigma-Aldrich #19160) which measures the percentage of a water-soluble tetrazolium salt (WST-1) formazan complex inhibition. The protein content of the enzyme extract was measured using Bradford reagent (Sigma-Adrich

#B6916) to express SOD activity as U mg protein^{-1}. The CAT activity was determined by the method of Clairborne (1985), where H_2O_2 decomposition was determined, and one unit of enzyme activity was defined as the amount of enzyme required to degrade 1 mmol of H_2O_2 in 1 min.

Histological analysis

Davidson preserved scallops from each treatment and sampling were dehydrated, embedded in Paraplast XT (SPI Supplies, West Chester, PA, USA), cut at 4 µm sections, mounted in glass slides and stained with hematoxylin-eosin (Kim *et al*., 2006). From the hematoxylin-eosin stain, three randomly selected digital images taken at 20x were processed with Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) in three tissues (smooth and striated muscles and digestive gland). The information was used to determine the gross physiological condition of juveniles using digestive gland index (DGI), smooth muscle index (SmMI) and striated muscle index (StMI) as described by Mazón-Suástegui *et al*., (2009). The following formula calculated the DGI:

$$\mathrm{DGI}\ (\%) = (\mathrm{CAA} / \mathrm{TCA}) \times 100 \qquad (1)$$

where CAA is the coverage area of the individual adenomeres (µm^2), and TCA is the total coverage area of the image (µm^2).

Muscular indexes (SmMI, StMI) were determined by the formulae:

$$\mathrm{SmMI}(\%) = (\mathrm{CAIM}/\ \mathrm{TCA}) \times 100 \qquad (2)$$

$$\mathrm{StMI}(\%) = (CAIM/\ TCA) \times 100 \qquad (3)$$

where CAIM is the coverage area of individual muscle-fiber packages (µm^2), and TCA is the total coverage area of the image (µm^2).

Biochemical analyses

Biochemical composition of soft tissues was assessed by triplicate. Samples previously stored at -80°C were lyophilized, re-hydrated in 1 mL cold distilled water, and homogenized to obtain crude extracts that were then used to determine carbohydrate, protein, and lipid contents. Determination of total proteins was done using the method of Smith *et al*. (1985), which uses a BCA reagent (Sigma-Aldrich #B9643) and bovine serum albumin (Sigma-Aldrich #9048-46-8) as the standard; absorbance was read at 562 nm. Carbohydrate content was determined according to Roe *et al*. (1961), using a reagent blank and dextrose solution as the standard (Vedco #3803); absorbance was read at 630 nm. The lipid content in tissues was determined by the sulpho-phospho-vanillin method following a modified version of Barnes & Blackstock (1973) using a microplate with 20µL supernatant extract previously digested with sulphuric acid and 200 µL lipid reactive solution (phosphor-vanillin 0.2% and sulphuric acid 80%). A mix of triglycerides and cholesterol (Sigma-Aldrich Lin-Trol B-1153) was used as a standard; absorbance was recorded at 540 nm.

Growth and survival

Growth and survival of scallops during the experiment were evaluated every week for 21 days by sampling 30 ind per replicate. Survival was assessed identifying organisms as either alive or dead and removing dead organisms from the experimental units to prevent poor water quality. Growth in length was assessed taking a sample of each unit and digital images were taken to measure organisms shell length using the program Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

For each triplicate, data normality was initially analyzed with the Kolmogorov-Smirnov test and then confirmed with the Levene test for homogeneity of variances. For histological analysis percentage values were transformed using arcsine (Zar, 2010). After that, one-way analyses of variance (n = 3, triplicate experimental units) were run to assess significant differences in the histological indexes, biochemical composition and enzymatic tissue activity between treatments. As needed, post-hoc multiple range mean comparisons with Tukey's test (HSD) were performed. The level of significance was set at $P < 0.05$ for all analyses. All data were analyzed using the program Statistica® 10.0 software (StatSoft, Tulsa, OK, USA) and expressed as mean ± 95% confidence interval.

RESULTS

Influence of homeopathic drugs on hemocyte count

The hemocyte count in digestive gland tissue showed a marked effect of homeopathic treatments. The counts for organisms from treatments T1 (44), T2 (41) and T3 (36) were significantly higher ($P < 0.05$) than organisms at T_o and all control groups (16-34) (Fig. 1).

Influence of homeopathic drugs on enzymatic biochemistry

Superoxide dismutase (SOD)

The highest SOD activity in muscle ($P < 0.05$) was observed at the T_o (11 U mg protein^{-1}) compared to all experimental groups (8-9 U mg protein^{-1}) except for control group C2 (10 U mg protein^{-1}) which was formulated with diluted and succussed ethanol (Fig. 2a).

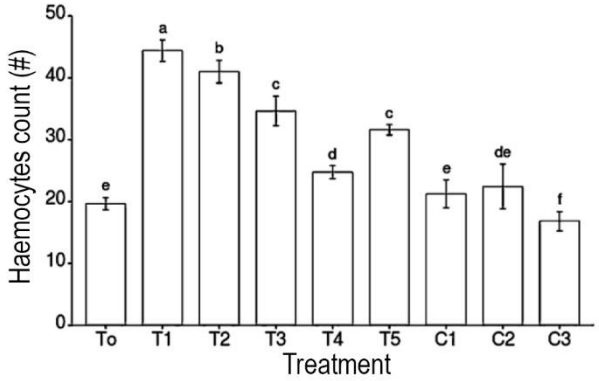

Figure 1. Total hemocyte count of *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters denote significant differences ($P < 0.05$).

In digestive gland, the SOD activity was significantly ($P < 0.05$) activated in all homeopathic treatments (7-8 U mg protein^{-1}) compared to the T_o (5 U mg protein^{-1}) and all control groups (5 U mg protein^{-1}) (Fig. 2b). The activity of SOD in gills was higher ($P < 0.05$) than in the other tissues and was significantly activated ($P < 0.05$) by homeopathic treatments T2 (37 U mg protein^{-1}) compared with T_o and the rest of the experimental groups (26-32 U mg protein^{-1}) with the exception of T5 (35 U mg protein^{-1}) (Fig. 2c). The SOD activity in mantle significantly increased ($P < 0.05$) in individuals treated with homeopathic treatment T5 (21 U mg protein^{-1}) compared to T_o and the rest of the experiment groups (16-19 U mg protein^{-1}) except control group C2 (20 U mg protein^{-1}) (Fig. 2d).

Catalase

The CAT activity in muscle was significantly higher ($P < 0.05$) in organisms from T5 (20 nmol min^{-1} mL^{-1}) and C1 (23 nmol min^{-1} mL^{-1}) than T_o and the rest of the experimental groups (0.73-10 nmol min^{-1} mL^{-1}) (Fig. 3a). In digestive gland, CAT activity was higher ($P < 0.05$) in homeopathic treatments T3 (30 nmol min^{-1} mL^{-1}) and T5 (21 nmol min^{-1} mL^{-1}) than T_o and the rest of the experimental groups (4-15 nmol min^{-1} mL^{-1}) (Fig. 3b). In gills, CAT activity was higher ($P < 0.05$) in organisms from control group C1 (21 nmol min^{-1} mL^{-1}), formulated with diluted ethanol, and C2 (20 nmol min^{-1} mL^{-1}) formulated with ethanol diluted and succussed compared to T_o and the rest of the experimental groups (3-14 nmol min^{-1} mL^{-1}) (Fig. 3c). The highest ($P < 0.05$) CAT activity was seen in mantle compared to the other tissues and registered the highest values ($P < 0.05$) in organisms from homeopathic treatments T1 (58 nmol min^{-1} mL^{-1}), T2 (51 nmol min^{-1} mL^{-1}) and T3 (47 nmol min^{-1} mL^{-1}) compared to T_o and the rest of the experimental groups (3-23 nmol min^{-1} mL^{-1}) (Fig. 3d).

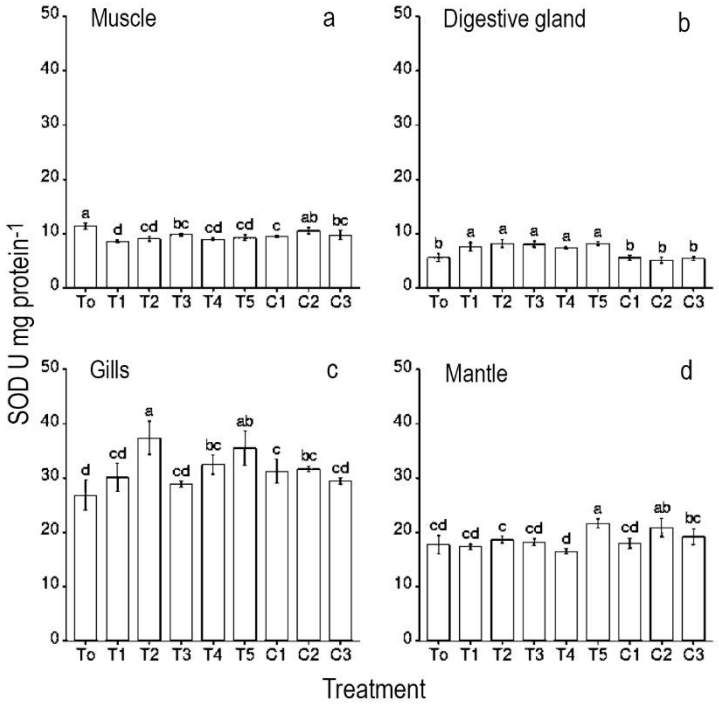

Figure 2. Superoxide dismutase enzyme activity (SOD) in a) muscle, b) digestive gland, c) gills, and d) mantle of *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters denote significant differences ($P < 0.05$).

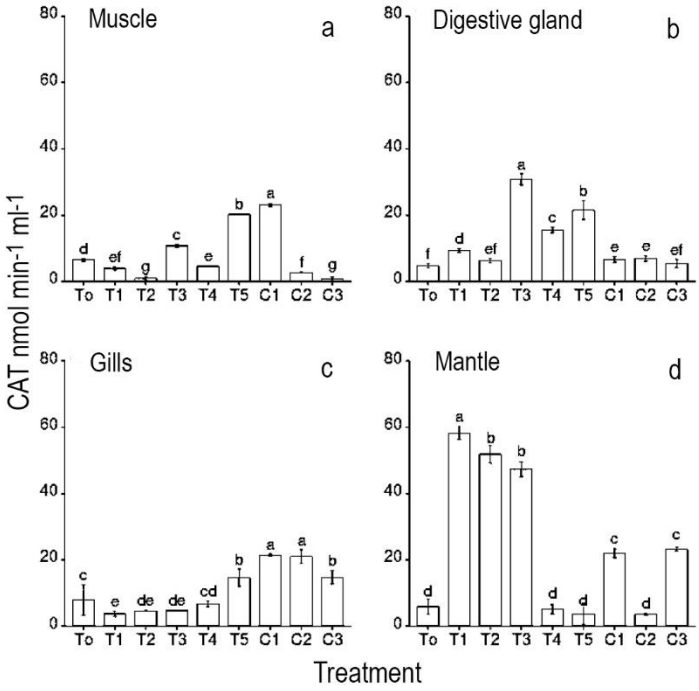

Figure 3. Catalase enzyme activity (CAT) in: a) muscle, b) digestive gland, c) gills, and d) mantle of *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters denote significant differences ($P < 0.05$).

Influence of homeopathic drugs on the histological index: smooth muscular index, striated muscular index, and digestive gland index

The SmMI value of all experimental groups (67-73%) was higher ($P < 0.05$) than T_o (62%) except for T3 (64%) (Fig. 4a). The StMI showed a significantly higher value ($P < 0.05$) in individuals from treatments T4 (77%) and T5 (76%) compared to T_o and the rest of the experimental groups (67-73%) (Fig. 4b). Significant differences ($P < 0.05$) were observed in DGI between groups. All experimental groups were significantly higher (45-52%) than T_o of the experiment (38%) (Fig. 4c).

Influence of homeopathic drugs on biochemical composition

Total carbohydrates

Carbohydrate concentration in muscle was higher ($P < 0.05$) in the organism from treatment T4 (50 mg g^{-1}) than T_o and the rest of the experimental groups (17-37 mg g^{-1}). The digestive gland was the tissue with the highest ($P < 0.05$) carbohydrate content and showed the highest ($P < 0.05$) values in organisms from treatment T3 (126 mg g^{-1}) and T5 (126 mg g^{-1}) compared to T_o and the rest of the experiment groups (68-125 mg g^{-1}). In gills, carbohydrate content was higher ($P < 0.05$) in organisms from C1 (14 mg g^{-1}) than T_o and the rest of

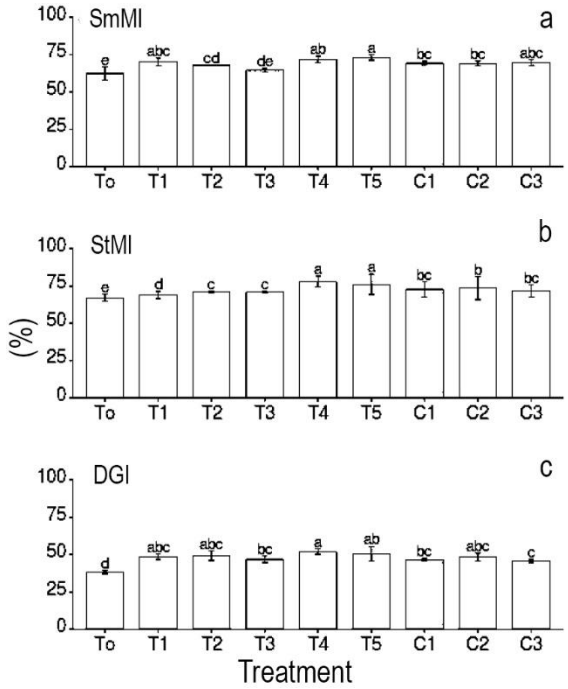

Figure 4. a) Smooth muscle index (SmMI), b) striated muscle index (StMI), and c) digestive gland index (DGI) of *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters denote significant differences ($P < 0.05$).

Table 1. Total carbohydrate, protein and lipid content in muscle, digestive gland, gills and mantle in *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters in the same raw denote significant differences ($P < 0.05$).

	To	T1	T2	T3	T4	T5	C1	C2	C3
Charbohydrates (mg g^{-1})									
Muscle	17 ± 2.9^{e}	37 ± 0.001^{b}	31 ± 0.14^{bc}	36 ± 1.5^{b}	50 ± 3.1^{a}	23 ± 0.5^{de}	36 ± 4.6^{b}	36 ± 8.7^{b}	25 ± 2.9^{cd}
Digestive gland	68 ± 0.7^{e}	117 ± 2.4^{d}	118 ± 3.6^{cd}	126 ± 1.6^{a}	121 ± 4.6^{bcd}	126 ± 1.1^{a}	122 ± 4^{abc}	125 ± 0.9^{ab}	117 ± 1.3^{d}
Gill	12 ± 0.2^{cd}	13 ± 0.2^{b}	11 ± 0.2^{d}	12 ± 0.4^{c}	10 ± 0.4^{f}	12 ± 0.04^{cd}	14 ± 0.2^{a}	11 ± 0.03^{e}	10 ± 0.5^{e}
Mantle	15 ± 0.1^{a}	13 ± 0.5^{b}	12 ± 0.2^{bc}	13 ± 0.2^{b}	14 ± 0.3^{a}	12 ± 0.4^{b}	13 ± 0.7^{b}	11 ± 0.7^{c}	12 ± 0.3^{b}
Protein (mg g^{-1})									
Muscle	561 ± 3.5^{a}	492 ± 16^{b}	448 ± 12^{c}	410 ± 1^{ef}	400 ± 10.8^{f}	421 ± 14^{de}	434 ± 16^{cd}	421 ± 0.8^{de}	452 ± 5.6^{c}
Digestive gland	479 ± 10^{a}	391 ± 6^{b}	368 ± 44^{bc}	320 ± 4.1^{efg}	300 ± 4.3^{g}	311 ± 1.9^{fg}	361 ± 13^{cd}	335 ± 1.7^{def}	347 ± 7.5^{cde}
Gill	390 ± 0.8^{a}	281 ± 3^{bc}	296 ± 29^{b}	260 ± 2.7^{de}	249 ± 9.2^{ef}	243 ± 4^{ef}	276 ± 2.9^{cd}	240 ± 4.4^{f}	235 ± 2.4^{f}
Mantle	486 ± 5^{a}	314 ± 4.5^{d}	403 ± 0.9^{b}	348 ± 8.2^{c}	285 ± 4.3^{d}	506 ± 9.8^{a}	424 ± 20^{b}	480 ± 21^{a}	409 ± 38^{b}
Lipids (mg g^{-1})									
Muscle	3 ± 0.01^{f}	19 ± 1.3^{b}	16 ± 0.4^{c}	18 ± 1.1^{bc}	22 ± 1.6^{a}	7 ± 0.4^{e}	18 ± 0.8^{bc}	13 ± 0.1^{d}	17 ± 2.4^{c}
Digestive gland	11 ± 0.2^{e}	58 ± 1.4^{d}	85 ± 5.3^{a}	75 ± 1.6^{b}	82 ± 1.5^{ab}	51 ± 0.2^{d}	56 ± 2.8^{cd}	68 ± 2.3^{c}	55 ± 9.5^{d}
Gill	2 ± 0.4^{f}	23 ± 0.02^{b}	21 ± 1.2^{d}	30 ± 1.4^{a}	20 ± 0.9^{de}	23 ± 0.5^{bc}	19 ± 1.5^{e}	29 ± 0.4^{a}	21 ± 0.9^{cd}
Mantle	5 ± 0.03^{f}	20 ± 1.8^{cd}	19 ± 1^{d}	22 ± 1.3^{bc}	26 ± 2.7^{a}	15 ± 0.1^{e}	22 ± 0.7^{bc}	24 ± 1.3^{ab}	27 ± 1.7^{a}

the groups (10-13 mg g^{-1}). The mantle showed a higher carbohydrate content ($P < 0.05$) in organisms in T_o of the experiment (15 mg g^{-1}) and T4 (14 mg g^{-1}) compared to the rest of the experimental groups (11-13 mg g^{-1}) (Table 1).

Total proteins

The muscle was the tissue with the highest ($P < 0.05$) protein content. Individuals from T_o (561 mg g^{-1}) had the highest content ($P < 0.05$) compared to all experimental groups (400-492 mg g^{-1}). Same as in muscle, the digestive gland and gill showed the highest protein content ($P < 0.05$) in individuals at T_o of the experiment (479 mg g^{-1}, 390 mg g^{-1}, respectively) than the rest of the experimental groups (300- 391 mg g^{-1}, 235-296 mg g^{-1} respectively). In mantle, the protein content was significantly different. The highest ($P < 0.05$) values were observed in individuals from T_o (486 mg g^{-1}), T5 (506 mg g^{-1}) and C2 (480 mg g^{-1}) compared to the rest of the experimental groups (285-424 mg g^{-1}) (Table 1).

Total lipids

The individuals from the homeopathic treatment T4 (22 mg g^{-1}) showed a higher lipid content in muscle ($P < 0.05$) than T_o and the rest of the groups (3-19 mg g^{-1}) (Table 1). Digestive gland, the tissue with the highest lipid content ($P < 0.05$), showed the highest values ($P < 0.05$) in individuals from homeopathic treatments T2 (85 mg g^{-1}) and T4 (82 mg g^{-1}) compared to T_o and the rest of the experimental groups (11-75 mg g^{-1}). The lipid content in gills was significantly higher ($P < 0.05$) in T3 and C2 (30 mg g^{-1}, 29 mg g^{-1}) than T_o and the rest of the experimental groups (2-23 mg g^{-1}). In mantle, the highest lipid content ($P < 0.05$) was observed in control C3 (27 mg g^{-1}) and T4 (26 mg g^{-1}), and the lowest at T_o (5 mg g^{-1}), T1 (20 mg g^{-1}), T2 (19 mg g^{-1}), T5 (15 mg g^{-1}) and C1 (22 mg g^{-1}) (Table 1).

Influence of homeopathic drugs on survival and growth rate

Survival was significantly higher ($P < 0.05$) in organisms from treatments T3 (95%) and T5 (94%) compared to C1 (88%) and C3 (85%) (Fig. 5a). Nevertheless, no significant differences were observed between T3 (95%) and T5 (94%), when compared to the control C2 (93%). The growth rate in shell length showed an effect by homeopathic treatment. Treatments T1 (117 µm) and T2 (108 µm) had the highest increase in length ($P < 0.05$) and were significantly different from controls C1 (14 µm), C2 (34 µm) and C3 (20 µm) (Fig. 5b).

DISCUSSION

This study revealed that homeopathic treatment formulated from pathogenic bacteria at decimal and centesimal dilutions (1D, 7C) had the potential to activate immunomodulatory response and antioxidant system of the scallop *Argopecten ventricosus*, accompanied with increases in growth, followed by those formulated from sodium metasilicates-phosphoric acid and Vidatox® as it is described below. Several authors have reported that homeopathic treatments work on the immune system (Bellavite *et al.*, 2006, 2014b; Bell & Schwartz, 2015). Particularly in marine organisms, homeopathic treatments from pathogenic bacteria, Vidatox®, and phosphoric acid, have been observed

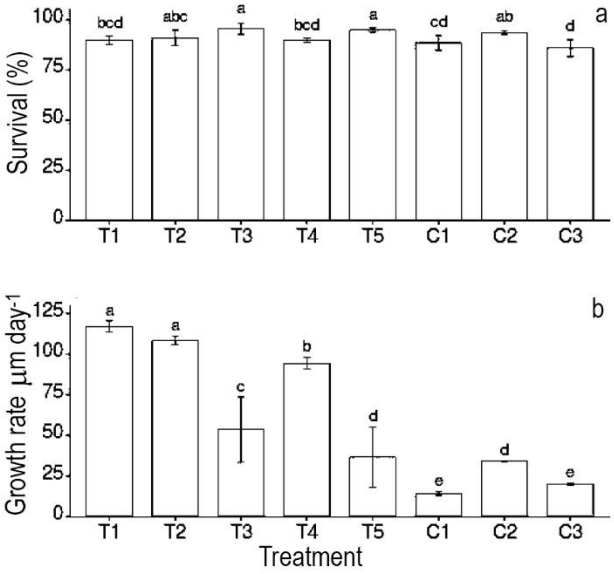

Figure 5. a) Survival and b) growth rate of *Argopecten ventricosus* juveniles treated with homeopathic drugs for 21 days. Values are mean ± confidence intervals at 95%. Different letters denote significant differences ($P < 0.05$).

to produce an immunomodulatory response (Mazón-Suástegui *et al.*, 2017; Ortiz-Cornejo *et al*., 2017).

In marine bivalves, hemocytes can be found in tissues because had an open circulatory system (Beninger & Le Pennec, 2006), and some authors have found differences at hemocytes count only in tissue and not in hemolymph (Cochennec-Laureau *et al*., 2003). We propose to measure the hemocytes count in the digestive gland tissue which showed higher hemocyte proliferation compared to other tissues. As hemocytes migrates to the tissues where danger signal are recognized, it was assumed that homeopathic treatments compounds are mainly recognized at digestive gland tissue; however, more investigation must be done to confirm this idea.

Haemocyte count was highly influenced by the homeopathic treatments formulated from pathogenic bacteria, followed by sodium metasilicate-phosphoric acid and Vidatox®. Homeopathic treatment formulated by pathogenic bacteria showed the high hemocytes counts which were one to three times higher than controls (C3, C2, and C1). Increases of hemocytes 1.4 times higher compared with controls have been previously related to the activation of the immune response in the clam *Ruditapes philippinarum* and *R. decussatus* (Oubella *et al*., 1993). Also, increases of these immune related cells have demonstrated to improve immune response when organisms are a challenge against pathogens (Prado-Alvarez *et al*., 2015). The increase in hemocyte count has been considered as an indicator of the activation of the immune system response since they are the main cellular effectors and act as defense mechanisms in bivalves (Canesi *et al*., 2002; Song *et al*., 2010). The fact that homeopathic treatment formulated by pathogenic bacteria allowed an increase in the haemocyte count could be supported by the presence of diluted pathogenic compounds (*e.g*., toxins, lipopoly-saccharides, beta glucans) from *Vibrio* cells and the recognitions of specific epitopes, which are the part of the antigen recognized by the immune system.

Increase in hemocyte proliferation had been associated to the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which are molecules involved in the recognition process that triggers immune system reaction (Song *et al*., 2010; Wang *et al*., 2013), but toxins (Prego-Faraldo *et al*., 2017) and nanoparticles (Moyano *et al*., 2012) can also be detected as danger signals and stimulate immune response. In very high diluted homeopathic treatments, the presence of nanoparticles from the initial molecules has been detected (Chikramane *et al*., 2012; Bell & Schwartz, 2015). Homeopathy effect has been attributed to a response of a low intensity silent danger signal, involving nanoparticles and high diluted compounds, where endogenous amplification may involve hormesis, time-dependent sensitization and stochastic resonance (Bell *et al*., 2013). Some authors have successfully improved organism health and resistance to disease using the same bacteria to formulate the homeopathic treatment (Camerlink *et al*., 2010; Mazón-Suástegui *et al*., 2017; Ortiz-Cornejo *et al*., 2017), although most studies that have proven immune stimulation with bacteria have been carried out using inactivated pathogenic strains (Balseiro *et al*., 2013; Pauletto *et al*., 2014). This study supports the effect of the homeopathic drug formulated by pathogenic bacteria at low and high dilutions, as both dilutions 1D (1×10^{1}) and 7C (1×10^{14}) promotes an increase of hemocytes count. The effects of these homeopathic treatments were attributed to micro-particles (*e.g*., epitopes) or highly diluted compounds from pathogenic bacteria that can trigger an immune response. It must be noticed that immunostimulants effect had been attributed to PAMPS like peptoglican and lipopoly-saccharides, however, the application of homeopathic treatment formulated by bacteria, which are un-expensive and may imply a complex diversity of high diluted epitopes, had never been evaluated in *A. ventriocosus*.

The immune activation caused by the homeopathic treatments from sodium metasilicate-phosphoric acid and Vidatox® were in accordance to the results reported by Ortiz-Cornejo *et al*. (2017), and it is believed that these treatments could be worked by the effect of diluted compounds and nanoparticles as well. Scarce

information is available about homeopathic treatments from sodium metasilicates, but at non-homeopathic concentrations, they have shown anti-inflammatory properties in murine macrophages and proved effectiveness in scavenging free radicals when used at 1, 5, 10 and 50 µM concentrations (Kim *et al*., 2013), a concentration close to our 1D dilution. In rats, the addition of sodium metasilicates has been associated to a beneficial action because silica in physiological amounts influences the complex network of cytokine interaction that regulates the immune response (Nielsen, 2008), while the intake of phosphoric acid has been associated to an increase of phosphorus in the organisms (Caravati, 1987). Phosphorus in high concentrations can promote decalcification and decrease growth (Calvo & Tucker, 2013), but it can improve health in moderate concentration. In hybrid sturgeons, the addition of phosphorus in the diet at 0.63 and 1.15% activates antioxidant enzymes and promotes eradication of free radicals (Jin *et al*., 2012). In homeopathic concentrations, phosphoric acid has been related to the improvement of the immune response in Catarina scallop (Mazón-Suástegui *et al*., 2017). The interaction between sodium metasilicates and phosphoric acid at homeopathic dilution 1D proved to increase immune response in *A. ventricosus* juveniles, which agrees with literature. Vidatox® (Havana, Cuba) has also demonstrated to increase hemocyte count. It is a registered trademark of a commercial homeopathic drug from the scorpion *Rhopalurus juneceus* venoms and sold to treat cancer in humans (http://vidatox-romania.ro/en/what-is-vidatox/).

Nonetheless, Giovannini *et al*. (2017) observed that it could induce hepatocellular carcinoma proliferation in culture mouse cells. Thus, it has to be handled carefully because these authors used the commercial solution Vidatox®, which is a hydroalcoholic solution (30°GL), and carcinoma proliferation could be an effect of the alcohol itself. It has been reported that ethanol can suppress mouse macrophages, immune response (Watson *et al*., 1988), inhibit TLR4 pathway and decrease cytokines (Pruett & Fan, 2009). In order to avoid the undesirable effects of ethanol, distilled water was used as a final excipient to formulate homeopathic treatments in this study. Although our results suggested Vidatox® dynamized 1C in distilled water can activate the immunomodulatory response.

The increase in hemocyte count in organisms treated with homeopathy formulated from pathogenic bacteria, sodium metasilicate-phosphoric acid, and Vidatox® were mainly related to increases in the antioxidant enzymes CAT and SOD activity. The former supports the idea of the immune system activation as increases of expression of antioxidant enzymes accompanied by an increase of hemocytes have already been linked in the oyster *Ostrea edulis* to immune response activation (Prado-Alvarez *et al*., 2015). The highest SOD and CAT activities occurred as a response to overcome oxidative stress in a better way when organisms are subjected either to environmental factors, pollutants or pathogen infection (Vlahogianni *et al*., 2007; Song *et al*., 2010). SOD and CAT can transform cytotoxic compounds produced by reactive oxygen species (ROS) as superoxide and hydrogen peroxide, in products not harmful for the cells such as water and oxygen (Song *et al*., 2010). In this study, treatments from pathogenic bacteria and sodium metasilicate-phosphoric acid showed a notorious activation of the antioxidant system (around the 300-500% of CAT activity in the mantle and about the 160% of SOD in digestive gland compared to controls). These treatments have been associated with antioxidant system activation at homeopathic and non-homeopathic solutions. Increases of CAT and SOD in digestive gland (around 600 and 250%, respectively) activities have been reported as an effect of toxin exposure (Prego-Faraldo *et al*., 2017) and presence of bacterial compounds (Song *et al*., 2010; Wang *et al*., 2013). Particularly CAT activity has demonstrated to contribute to the redox balance in *Chlamys farreri* and *Meretrix meretrix* to counter-react oxidative stress produced by pathogen attack (Li *et al*., 2008; Wang *et al*., 2013), and heat killed pathogenic bacteria, such as *V. splendidus* and *V. anguillarum* in *Mytilus galloprovincialis* which registered and increase between 58 to 98% of CAT activity compared to the control (Canesi *et al*., 2010). In mouse brain from organisms intoxicated with aluminum, an increase in transcription of the antioxidant enzymes SOD and CAT have been reported when silicic acid at 0.9% was administered to mice (Gonzalez-Muñoz *et al*., 2008). Evidence has been reported that proves the presence of silicic acid in homeopathic treatments by the effect of succussion of silicon products (Anick & Ives, 2007). Also, Mazón-Suástegui *et al*. (2017) reported an increase of the 10% in SOD activity when *A. ventricosus* juveniles were treated with homeopathic phosphoric acid compared with ethanol control. These slight increases in SOD activity promoted a higher survival of scallops during challenge assays with *Vibrio alginolyticus.* The activation of antioxidant enzy-mes strengthened organisms versus oxidative stress, which may help to overcome stressful conditions as disease. The results attained in this research prove that homeopathic treatments, particularly those formulated from pathogenic bacteria, are capable of activating the immune response at the cellular level and the antioxidant system of the Catarina scallop juveniles.

The commercial homeopathic treatments from Silicea terra and Phosphoric acid (Similia®) did not show a definite effect on the immune response, but they showed clear and significant increases of energetic reserves in treated scallops. This treatment promoted the accumulation of carbohydrates (mantle, muscle) and lipids (digestive gland, muscle). Carbohydrate reserves in mollusk bivalves are mainly related to muscle and lipid reserves to digestive gland (Maguire *et al*., 1999; Darriba *et al*., 2005). Both tissues (muscle and digestive gland) have been reported as the principal storage organs of energetics reserves (Barber & Blake, 2006). Protein levels depletion (around 12 to 41%) in all experimental groups was related to overall nutrition conditions in the laboratory, which allowed storing energetic reserves. The depletions of protein levels have been associated with an increase in other energetic reserves like carbohydrates and lipids (Freites *et al*., 2003). As all treatments had the same diet, an increase of reserves by Silicea terra and phosphoric acid (Similia®) could be related with an enhancement in food assimilation processes. Homeopathic Silicea terra has been reported as a growth promoter in *A. ventricosus* (Mazón-Suástegui *et al*., 2017). In the non-homeopathic presentation, silicon dioxide at 0.02% in turkey diet increased growth and reduced nitrogen loss (Tran *et al*., 2015). Phromkunthong (2015) reported that Silica+, a micronized silica powder, increased peptidases accompanied with an increase in growth in the shrimp *Litopenaeus vannamei.* In the case of phosphoric acid (Similia®) homeopathic solutions are recommended to improve food assimilation. More evaluation between silica-phosporic acid and digestive enzyme should be done to elucidate if treatments were acting on enzymatic activity to promote nutrient intake or by other via. The accumulation of reserves by Silicea terra and phosphoric acid treatments may contribute to having organisms more resistant to stressor factors as a disease caused by pathogenic bacteria may cause a depletion of energy reserves (Genard *et al*., 2013).

The DGI and SmMI did not show a specific effect by treatment, but StMI was higher in scallops treated with Silicea terra and phosphoric acid (Similia®) although this treatment registered the highest carbohydrates content in muscle. StMI increase could be a consequence of the accumulation of metabolic reserves, mainly carbohydrates. As discussed above, Silicea terra and phosphoric acid (Similia®) treatment were associated to promote better assimilation. It should be noticed that muscle is one of the main carbohydrates reserves in scallops and striated muscle can store a higher content of carbohydrates than smooth muscle (Maguire & Burnell, 2001).

As all treatments receive the same diets and hatchery conditions were kept at overall conditions, growth was attributed to treatment effect. A relationship was observed between growth in shell length and the homeopathic treatments formulated by pathogenic bacteria, which was unexpected as treatments from Silicea terra-phosphoric acid have shown a significant increase in energetic reserves and muscular condition index. Treatments with the higher growth rate in length showed higher hemocyte count. It has been observed that hemocytes contribute to wound and shell repair (Beninger & Le Pennec, 2006), playing an important role in calcium transport to shell regeneration (Kádár, 2008). Haemocytes count has been associated with induction to shell repair (Mount *et al*., 2004). This finding makes us suppose that hemocytes could have contributed to shell formation and could be helping to increase shell calcification and growth as in condition index were minimal differences between most of the treatments; more studies should be required to corroborate this information.

CONCLUSIONS

In conclusion, the homeopathic treatments, mainly those formulated from pathogenic bacteria prove to generate a quantifiable biological effect, increasing hemocyte count and antioxidant enzyme activity. Homeopathic treatments formulated by Silicea terra-phosphoric acid contributed to energetic reserve accumulation in the principal storage tissues. Homeopathic treatments are a good alternative to activate the immune system at the cellular level and the antioxidant system because of their easiness for preparation, storage, and administration. Homeopathy for aquaculture is a novel and eco-friendly prophylactic option to improve the overall condition of *A. ventricosus* juveniles at the pre-fattening stage.

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