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Research Article

Oyster (*Crassostrea gasar*) gastrointestinal tract microbiota and immunological responses after antibiotic administration

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ABSTRACT. Microbiota, the microorganisms that colonize living organisms. Oysters rely exclusively on an innate immune system. This study characterized the cultivable gastrointestinal tract microbiota (GTM) of the oyster *Crassostrea gasar* and evaluated their influence on immune responses. Antibiotics were used to reduce or alter the microbiota during *in vitro* and *in vivo* assays. Haemocyte viability, concentration, and phagocytic capacity, as well as the production of reactive oxygen species (ROS), were evaluated in antibiotic-treated and control oysters. Microbiological analysis of the gastrointestinal tract was also performed; bacteria were selected using culture media and were identified by 16S ribosomal DNA. The results showed that the antibiotics eliminated bacteria *in vitro* but increased their concentrations *in vivo*. The GTM was composed of 26% amylolytic bacteria, 21% lipolytic bacteria, 18% proteolytic bacteria, 18% cellulolytic bacteria and 17% lactic acid bacteria; there were no differences in the amounts of these bacterial types between the control and treated oysters. Three major bacterial phyla, Proteobacteria, Firmicutes and Actinobacteria, and seven genera, *Labrenzia*, *Pseudomonas*, *Halomonas*, *Shewanella*, *Vibrio*, *Bacillus*, and *Micrococcus,* were detected. The concentration, viability, and phagocytic capacity of hemocytes and the production of ROS were not significantly altered by antibiotic treatment. In conclusion, the antibiotics altered the number of heterotrophic bacteria without changing hemocyte function, suggesting that this assay could be useful for verifying the influence of microbiota in host-parasite interactions.

Keywords: *Crassostrea gasar*; bacterial community; bivalves; hemocytes; phagocytosis; reactive oxygen species; 16S rDNA

INTRODUCTION

The number of studies on the role of microbiota in living organisms has increased in recent years. In humans, intestinal microbiota contributes to improving the performance of the immunological system (Wu & Wu, 2012; Belkaid & Hand, 2014). In invertebrates, there is similar evidence of the benefits of resident intestinal microbiota for the organisms' health and resistance to pathogens (García-García *et al*., 2013). In aquatic organisms, especially fish and crustaceans, bacteria have been widely investigated and have been successfully employed as probiotics in larvae, juveniles and adults for disease control in production systems. Such studies have shown the clear benefits of probiotics for growth, resistance to pathogens and immunological responses, which enables the reduction and/or elimination of antibiotic administration (Newaj-Fyzul *et al*.,

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2014). In bivalves, probiotic use is less common and is limited to the first phases of life (in hatchery facilities), during which it is used to control pathogenic bacterial strains and to reduce the use of chemotherapeutic agents (Prado *et al*., 2010; Dubert *et al*., 2017).

Oysters are filter-feeding organisms that accumulate a rich variety of microbiota in their gastrointestinal tract. These microbiota are composed of representatives of many phyla, such as Firmicutes, Proteobacteria, Cyanobacteria, Spirochaetes, Planctomycetes, Verrucomicrobia, Fusobacteria, Tenericutes and Bacteroidetes (Green & Barnes, 2010; Fernandez-Piquer *et al*., 2012), as well as those of a wide diversity of bacterial genera, including *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus*, and *Bacillus* (King *et al*., 2012; Wegner *et al*., 2013; Romalde *et al*., 2014; Trabal-Fernández *et al*., 2014). *Vibrio* is one the most common genera of bacteria in seawater (Tonon *et al*., 2015), and it includes species that are pathogenic to the larvae and spat of bivalves (Dubert *et al*., 2017). Bivalves also have microbiota associated with their gills (Wegner *et al*., 2013). Notably, the gills and gastrointestinal organs of bivalves are considered entry sites for infectious and parasitic agents (Vasta, 2009; Cardinaud *et al*., 2014; Ben-Horin *et al*., 2015). Therefore, the presence of microbiota associated with these organs is believed to minimize and control infections.

One way to assess the role of microbiota in the physiological responses of an organism is by partially or entirely eliminating the bacterial community through antibiotic administration. Ridley *et al.* (2013) eliminated the bacterial load of *Drosophila melanogaster* by oral antibiotic administration (99% reduction) and egg dechorionation (bacteria were undetectable) and concluded that microbiota plays an important role in promoting fly development. In bivalves, no study for this purpose has been published to date, probably because of the difficulties in obtaining 100% bacteria-free spat. Nevertheless, some studies have evaluated the toxicity of antibiotics to mussel and clam hemocytes (Matozzo *et al*., 2015, 2016).

Hemolymph is a body fluid analogous to vertebrate blood that contains hemocytes, which are the backbone of the bivalve immune system. Hemocytes are responsible for a highly elaborate and efficient array of defense responses against infectious agents and parasites (Allam & Raftos, 2015; Bachère *et al*., 2015). Bivalve hemolymph also contains its own microbiota (Desriac *et al*., 2014; Lokmer & Mathias-Wegner, 2015). Hemocytes are also responsible for many physiological functions, such as shell formation, digestion, nutrient transport and wound repair (Cheng, 1996).

Native mangrove oysters (*Crassostrea gasar* and *C. rhizophorae*) are a source of income for local Brazilian communities. On the north-eastern coast of Brazil, fishermen, and fisherwomen commonly collect oysters from estuaries and sell them on the beaches for human consumption without sanitary control (Santos *et al*., 2017). In 2012, the federal government created the National Programme for Hygienic and Sanitary Control of Bivalve Molluscs (PNCMB), which states the rules for sanitary control in the production and trade of oysters, mussels, scallops, and cockles. Nevertheless, the state of Santa Catarina (in southern Brazil) is the only place that performs periodic microbiological and phytotoxin monitoring of cultured and extracted bivalves (de Souza *et al*., 2015). The production of native oysters is in development in northeastern Brazil, although a cultivated bivalve production chain is lacking (Lavander *et al*., 2013). *C. gasar* is currently the most promising native oyster species for cultivation in northern and northeastern (NE) Brazil because of its better performance in culture than *C. rhizophorae* (Scardua *et al*., 2017). Hatchery *C. gasar* spat production procedures have already been developed in a private enterprise (Da Silva *et al*., 2016). However, most of the oyster production in NE Brazil is already done using spat sampled from the natural environment (mangrove roots) or culture structures (ropes and bags) (Queiroga *et al*., 2015).

The present study aimed to characterize the bacterial community of the gastrointestinal tract of the oyster *C. gasar* and to understand its role in oyster immune defense responses.

MATERIALS AND METHODS

Oyster sampling

Between March and June 2016, *C. gasar* oysters (n = 340) with a shell height, *i.e.*, anteroposterior axis (Eble & Scro, 1996), of commercial size (7.2 ± 0,82 cm, mean ± SE (standard error)) were sampled from a suspended-fixed cultivation system located on the estuary of the Mamanguape River (06°47’08”S; 34°59’46”W). This system is constructed using wooden stakes fixed to the bottom of the river next to the river margin. Ropes are tied at the end of the stakes, forming a long line where plastic bags are hung. Oyster seeds (juveniles) are collected directly from the bottom of the river and deposited into the bags to grow until they reach marketable size (>70 mm in shell height).

Oysters were cleaned and washed with tap water before use. For *in vitro* assays, oysters (n = 40) were used immediately after sampling in order to better estimate the efficiency of the antibiotic mixture in

eliminating the total gastrointestinal tract bacterial load. For *in vivo* assays (n = 300), oysters were acclimated for 48 h in disinfected tanks containing 20 L of filtered (1 μm filter) and chlorinated (20 mg L^{-1} active chlorine) seawater at 25 of salinity in a closed system with constant aeration. This procedure was used to clean the intestine of faeces and to assist in the elimination of transient bacteria.

Antibiotic treatment of oysters

A microbiological approach was used to assess the effectiveness of a mixture of antibiotics in reducing or eliminating bacteria from the gastrointestinal tract of oysters in both *in vitro* and *in vivo* assays. This procedure used non-selective plate count agar medium (PCA, M091A, Himedia, Brazil) for the growth and quantification of total cultivable heterotrophic bacteria. This medium is composed of casein enzymic hydrolysate, yeast extract, dextrose, and agar and has a pH of 7.0.

The antibiotic mixture contained ampicillin (A0166, 3 μg mL^{-1}), erythromycin (E5389, 100 μg mL^{-1}), nystatin (N1638, 24 μg mL^{-1}), gentamicin (G1264, 50 μg mL^{-1}), kanamycin (K1377, 50 μg mL^{-1}), and chloramphenicol (C0378, 20 μg mL^{-1}) from Sigma-Aldrich, Brazil, and penicillin and streptomycin (15140122, 100 U mL^{-1} or 100 μg mL^{-1}) from Thermo Fisher Scientific, Brazil. The final concentrations were chosen based on the ranges recommended by the manufacturers.

In vitro assay

The gastrointestinal tracts of the oysters (n = 20) were aseptically excised and prepared in two pooled samples of 10 animals each. The samples were homogenized in filtered seawater at 10 of salinity (1:10 w v^{-1}) and serially diluted (1/10 to 1/100,000). The suspensions (1 mL) were distributed in Petri dishes in triplicate or duplicate using the pour plate method. Petri dishes contained PCA medium that was either supplemented with an antibiotic mixture (treated group) or not supplemented (control group). Plates were incubated for 48 h at 30°C, and colony-forming units (CFU) were counted and estimated as CFU g of $tissue^{-1}$. The assay was repeated twice.

In vivo assay

After acclimation, oysters were distributed in two tanks (n = 50 oysters/tank) to compose two groups: the treated group and the control group. Treated oysters received a daily dose of the antibiotic mixture inside the pallial (mantle) cavity (1 mL) through a shell hole for four days. The control group received sterile filtered seawater. The oysters remained out of the seawater for two hours before returning to their respective tanks to promote the assimilation of the antibiotics by the gastrointestinal epithelium and to prevent the immediate loss of the antibiotics in the tank seawater. Mantle cavity injection was chosen instead of intramuscular injection to allow the antibiotics to reach the gastrointestinal tract through the filtration and feeding path rather than reaching the circulatory system through the hemolymph. The assay was repeated three times.

The gastrointestinal tracts of the oysters (three pooled samples of 10 animals each) in the two groups were excised, prepared and analyzed as described above but without the antibiotic mixture in the PCA medium. The results are expressed as the mean CFU g of $tissue^{-1}$.

Bacterial isolation by metabolic ability

A single assay was performed, as described above, to characterize the microbiota after acclimation (control group) and antibiotic administration (treated group). The homogenates of the oyster gastrointestinal tracts were incubated in different culture media to select seven groups of bacteria.

Five bacterial groups were selected and classified according to their metabolic ability: bacteria capable of producing lactic acid (lactic acid bacteria) and those capable of degrading cellulose (cellulolytic bacteria), lipids (lipolytic bacteria), proteins (proteolytic bacteria) or starches (amylolytic bacteria). The following media were used as previously recommended by Gerhardt *et al*. (1994): de Man, Rogosa and Sharpe media (MRS, Difco, Brazil) for lactic acid bacteria and noncommercial media for cellulolytic, lipolytic, proteolytic and amylolytic bacteria. For *Bacillus* genus bacteria, PCA medium was used after heat treatment at 70°C; for *Vibrio* genus bacteria, thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco, Brazil) was used.

After bacterial growth, a second isolation procedure was performed in which 10 colonies of each bacterial group were selected based on morphological differences (size, shape, and color) between the colonies, yielding 420 samples in total (10 colonies × 7 media × 3 samples × 2 treatments). A sample from each colony was collected, and samples were deposited in microtubes containing the nonselective medium trypticase soy agar (TSA, Difco, Brasil) and incubated at 30°C for 48 h. Isolates that grew were characterized by their morphology (bacilli or cocci), size (short or long) and reaction to Gram staining. Isolates showing only one cell type were kept in the collection of bacterial strains.

Molecular characterization of bacterial isolates

Some bacterial isolates were selected for molecular characterization by sequencing the 16S ribosomal RNA

gene region (16S rDNA). Selection of a total of 93 isolates was performed to analyze approximately five different representatives from each bacterial group from the control and treated groups except for *Vibrio*, for which all isolates obtained were sequenced because of the importance of *Vibrio* bacteria as oyster pathogens (Beaz-Hidalgo *et al*., 2010; Travers *et al*., 2015; Le Roux *et al*., 2016; Dubert *et al*., 2017).

Genomic DNA extraction, amplification, and sequencing

The bacterial isolates (n = 93) were grown overnight in Luria-Bertani medium (LB, Difco, Brazil) and centrifuged (1000 *g*, 10 min), and then the supernatant was discarded. The cell pellet was subjected to genomic DNA extraction using the Wizard® SV Genomic DNA Purification System (Promega, Brazil) following the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed using the forward primer U968 (5' ACC GCG AAG AAC CTT AC 3') and the reverse primer L1401 (5' GCG TGT GTA CAA GAC CC 3') (Nubel *et al*., 1996; Gabor *et al*., 2004), which are specific for the V6-V8 region of the 16S rDNA. Each 12.5 µL reaction contained (in final concentrations) 1× proprietary buffer, 0.5 µL of DNA, 0.8 mM $MgCl_2$, 0.08 mM dNTPs, 0.08 µM of each primer and 0.5 U of Taq DNA polymerase (Invitrogen, Brazil). The PCR conditions included initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 8 min.

PCR products from the 93 bacterial isolates were directly sequenced in both directions by the Sanger method using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. The final volume of the sequencing reaction was 10 µL, which contained 0.8 µL of PCR product, 1 µL of 5× buffer, 2 µL of Big Dye solution and 3.2 pmol of primer. The sequencing products were purified with a standard isopropanol/ethanol protocol and read on an ABI 3500 genetic analyzer.

The obtained nucleotide sequences (16S rDNA) were compared to those registered in the Ribosomal Database Project (RDP, Michigan State University) and identified at the genus level (80% bootstrap confidence). The nucleotide sequences (n = 93) were deposited in the GenBank database under the accession numbers MH665890–MH665973 and MH685446–MH685454.

Effect of microbiota alteration on oyster immune cell parameters

Haemocyte parameters were evaluated in oysters from the *in vivo* assay. Hemolymph was withdrawn from the adductor muscle of oysters (n = 15 from each group) with a 1 mL syringe coupled to a 21-gauge needle and held on ice until use. Individual hemolymph samples were either 1) immediately fixed in formaldehyde (2% final concentration) for estimation of hemocyte concentration, or 2) deposited in cytometry tubes to separately measure cell viability, phagocytic capacity and the production of reactive oxygen species (ROS).

Haemocyte concentration in oyster hemolymph was assessed with the fluorescent DNA-intercalating stain SYBR®Green I (Molecular Probes, the final concentration of 10^{-4} from the original solution). Formalin-fixed hemocytes were gated on a green fluorescence *versus* complexity (side scatter, SSC) biplot. Cell concentrations were estimated considering sample dilution, cytometer flow and sample acquisition time (30 s) (Hégaret *et al*., 2003a). The viability of the hemocytes was assessed with the fluorescent DNA-intercalating stain propidium iodide (Sigma-Aldrich, final concentration 10 µg mL^{-1}). Cell mortality was estimated as the percentage of fluorescent (dead) cells among the total cells analyzed (Hégaret *et al*., 2003b). Phagocytosis was measured with fluorescent latex particles (Fluoresbrite® Yellow Green Microspheres, 2 µm; Polysciences, final concentration 0.1%, approximately 4.5×10^7 cells mL^{-1}). The phagocytic rate was estimated as the percentage of cells that engulfed one or more particles (Hégaret *et al*., 2003b). ROS were measured with 2′7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, final concentration 10 µM). The production of ROS was expressed as the geometric mean fluorescence of the hemocytes in arbitrary units (A.U.) (Hégaret *et al*., 2003b; Lambert *et al*., 2003).

Cell suspensions were analyzed after one hour incubation with fluorophores using a FACSCanto II flow cytometer (BD Biosciences, San Jose, California, USA). Data were analyzed with Flowing software (version 2.5.1, Turku, Finland).

Statistical analysis

Percentage data were transformed (by dividing the arcsine of the square root of the percentage by 100) for statistical tests but are presented as the untransformed percentage values. The Kolmogorov-Smirnov test was used to check the normality of the data. T-tests or Mann-Whitney (Wilcoxon) W-tests were used to compare the control and antibiotic-treated groups for all parameters studied. Differences were considered significant when $P \leq 0.05$. Statistical analyses were performed with Statgraphics Centurion XVI.II. The data are presented as the means ± standard error (SE).

RESULTS

Antibiotics eliminated or altered oyster gastrointestinal tract bacterial communities

No colonies grew on Petri dishes that received the medium supplemented with antibiotics; in contrast, control plates (without antibiotic supplementation) showed an average of $7.6 \pm 0.47 \times 10^6$ CFU g of tissue^{-1}. In the *in vivo* assay, the antibiotic mixture administered directly to oysters induced a significant increase (P = 0.004; Mann-Whitney test) in the CFU g of tissue^{-1} in the GTM ($4.6 \pm 0.62\times10^6$ CFU g of tissue^{-1}) compared to that of the control oysters ($2.4 \pm 0.36\times10^6$ CFU g of tissue^{-1}).

Bacterial characterization

Selective media isolated bacteria were belonging to five metabolic-related groups (cellulolytic bacteria, lactic acid bacteria, proteolytic bacteria, lipolytic bacteria, and amylolytic bacteria) and two genera (*Vibrio* and *Bacillus*). Comparisons between groups (treated and control) did not show differences in CFU g of tissue^{-1} for any bacterial group (Fig. 1).

The gastrointestinal tract microbiota of oysters contained similar relative proportions (calculated based on the mean of the logarithm of the CFU g of tissue^{-1}) of all the metabolic-related bacterial groups (data from both groups were combined): 26% amylolytic bacteria, 21% lipolytic bacteria, 18% proteolytic bacteria, 18% cellulolytic bacteria and 17% lactic acid bacteria.

The 16S rDNA sequences analyzed belonged to three phyla, four classes, seven families and seven genera. *Labrenzia* and *Halomonas* (phylum Proteobacteria) were found exclusively in the treated group, while *Shewanella* (phylum Proteobacteria) and *Micrococcus* (phylum Actinobacteria) were found exclusively in the control group (Table 1). Four sequences were not identified at the genus level; one was identified to be in the class Alphaproteobacteria, and the other three were identified at the family level (Pseudomoneaceae and Vibrionaceae) (Table 1). Out of the 28 sequences from TCBS-isolated bacteria, 1 corresponded to *Shewanella*, instead of *Vibrio.*

Alteration of oyster gastrointestinal tract bacterial communities did not modify hemocyte parameters

Haemocyte parameters were evaluated to verify whether changes in GTM would impair the immune cells and the immune responses of oysters. Haemocyte concentration, viability and phagocytosis were very similar between the oysters in both groups, while ROS production was lower in the antibiotic-treated oysters, although the difference was not significant at the 5% level (Table 2).

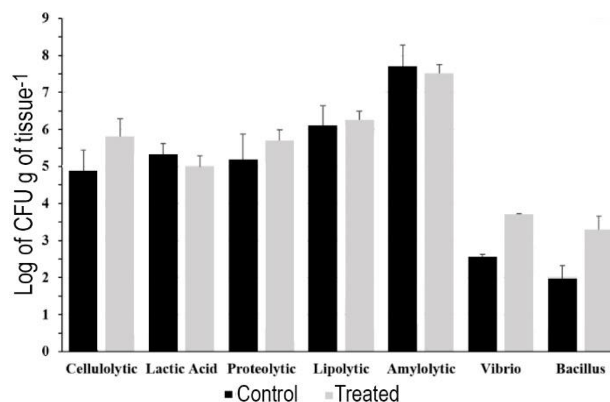

Figure 1. Quantities (log of CFU g of tissue^{-1}) of bacterial groups and genera isolated from the gastrointestinal tract of the oysters in the two groups. Treated: animals that received an intrapallial (mantle cavity) injection of an antibiotic mixture for four days. Control: animals that received sterile filtered seawater. No significant differences were observed between groups (t-test; n = 3). The data are presented as the means ± SE.

DISCUSSION

In the present study, the role of microbiota in the immune defense responses of *C. gasar* oysters was evaluated after the administration of antibiotics in the mantle cavity to eliminate or alter the bacterial community. The antibiotic mixture chosen has multiple bactericidal and/or bacteriostatic modes of action that affect several bacterial groups (Kohanski *et al*., 2010). The antibiotic mixture was effective *in vitro* since there was no heterotrophic bacterial growth. However, antibiotics administered in the oyster mantle cavity did not extinguish bacterial growth and likely promoted differentiated bacterial growth. The difference in outcomes between the two procedures can be explained by the direct contact of antibiotics in the solid agar medium with bacterial cells from the gastrointestinal tract homogenates in the *in vitro* assay.

In contrast, in the *in vivo* assay, antibiotics were assimilated by the natural process of feeding through the gills and digestive epithelia, which represent physical barriers for antibiotic intake and action. Moreover, we cannot exclude the possibility that antibiotics could have lost some of their efficacy due to salts, pH, the temperature of the seawater or even light exposure. Indeed, some antibiotics can be degraded by several physical and chemical conditions (Kümmerer, 2009a,b; Larsson, 2014). Furthermore, the exposure time to antibiotics (four days) might have been insufficient to cause a more significant decrease in the number of bacteria (Matozzo *et al*., 2015, 2016). We believed that administration of antibiotics for more than four days would compromise the measurement of the

Table 1. Identification of bacterial isolates (n = 93) by sequencing the 16S ribosomal RNA gene. The number of sequences obtained from control and antibiotic-treated groups that matched those of the Ribosomal Database Project (RDP, 80% bootstrap confidence) at the genus level is presented, except for four (unclassified) sequences that were identified at class (*) and family levels (**).

Phylum	Class	Family	Genus	Control	Treated	Total
Firmicutes	Bacilli	Bacillaceae	*Bacillus*	10	5	15
Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	*Labrenzia*	-	1	1
		Unclassified	Unclassified	-	1*	1
	Gammaproteobacteria	Vibrionaceae	*Vibrio*	23	38	61
			Unclassified	1**	1**	2
		Pseudomonadaceae	*Pseudomonas*	1	5	6
			Unclassified	-	1**	1
		Shewanellaceae	*Shewanella*	2	-	2
		Halomonadaceae	*Halomonas*	-	2	2
Actinobacteria	Actinobacteria	Micrococcaceae	*Micrococcus*	2	-	2
Total				39	54	93

Table 2. Comparison of hemocyte parameters between the control and antibiotic-treated groups. The data are presented as the means ± SE. *P*-values from t-tests or *Mann-Whitney tests. n = 45 oysters per treatment.

	Control	Treated	*P*
ROS production (A.U.)	1.513 ± 196	997 ± 92	0.1069*
Mortality (%)	5.8 ± 0.46	6.2 ± 0.59	0.5968
Cell concentration ($\times 10^5$ haemocytes mL^{-1})	5.1 ± 0.61	5.8 ± 0.57	0.3948
Phagocytic rate (%)	15.4 ± 0.98	16.2 ± 0.89	0.5033

immune cell parameters of oysters, *i.e.*, the effects could be a result of the antibiotics themselves and not the result of a change in the bacterial community.

The increase in total heterotrophic bacteria in the gastrointestinal tract of oysters submitted to antibiotics suggests a change in the bacterial community, which could be a result of a selective pressure established by the antibiotics. Bacterial strains that were more susceptible to antibiotic action could have been inhibited, favoring exacerbated growth of more resistant and proliferative strains (Kümmerer, 2009b; Martínez, 2009; Prado *et al*., 2010; Xiong *et al*., 2015). Despite the differences in total heterotrophic bacteria, the relative amounts of each bacterial group in the antibiotic-treated group were not different from those in the control group. When the medium is nonselective (as is PCA), the growth of most heterotrophic bacteria is favored, and the potential number of bacterial strains is much larger; thus, the selective pressure exerted by the antibiotics used could have produced significant differences in the CFU g of tissue^{-1} between the two groups. In contrast, when the medium is selective, a new barrier is added, making the potential number of bacterial strains smaller; thus, the amount of some bacterial groups in the treated group was not significantly higher than that of the control group despite the observed trend for *Vibrio*, *Bacillus* and cellulolytic bacteria. It is possible that a higher number of replicates (biological and technical) or a metagenomic approach (Neelakanta & Sultana, 2013) could have elucidated these differences.

The antibiotics seem to have caused modifications in the microbiota composition as well. The sequence data obtained from the treated and control groups corroborate this hypothesis because some genera were detected only in one of the groups. Seven genera and three bacterial phyla were identified; the phylum Proteobacteria was the most highly represented (including the genera *Labrenzia*, *Pseudomonas*, *Halomonas*, *Shewanella* and *Vibrio*), while representatives from Firmicutes and Actinobacteria were from only one genus (*Bacillus* and *Micrococcus*, respectively). Similar to the results of the present study, a previous study on the gastrointestinal microbiota of *Crassostrea hongkongensis* revealed *Vibrio*, *Pseudoalteromonas*, *Bacillus*, and *Shewanella* (Wang *et al*., 2016). In a similar study in *Crassostrea gigas*, *Vibrio* bacteria were detected in the highest abundance, followed by *Cytophaga*, *Alteromonas*, *Moraxella* and *Pseudomonas* bacteria (Iida *et al*., 2000), and *Ostrea edulis* had abundant *V. splendidus* and *V. harveyi*, although these bacteria were located throughout the

whole body (Pujalte *et al*., 1999). However, studies using metagenomic approaches have demonstrated that the gastrointestinal tract microbiota of oysters (*Crassostrea virginica*, *Crassostrea corteziensis*, *Crassostrea sikamea*, *C. gigas*, and *Saccostrea glomerata*) are much more diverse than previously thought (Green & Barnes, 2010; King *et al*., 2012; Trabal Fernández *et al*., 2014).

The selective media used, in this study, isolated total cultivable heterotrophic bacteria belonging to five metabolism-related groups (cellulolytic bacteria, lactic acid bacteria, proteolytic bacteria, lipolytic bacteria, and amylolytic bacteria) and two genera (*Vibrio* and *Bacillus*). These two genera are very important for aquatic organisms and, consequently, for aquaculture in general, by opposite reasons. *Vibrio* spp. are abundant in marine environments (Tonon *et al*., 2015), and some species are highly pathogenic to oysters (Beaz-Hidalgo *et al*., 2010; Romalde *et al*., 2014; Travers *et al*., 2015), including oysters in bivalve hatchery facilities (Dubert *et al*., 2017). In studies on the diversity of *Vibrio* associated with cultured bivalves in a natural environment or under hatchery conditions, the most abundant species were *V. splendidus*, *V. alginolyticus* and *V. harveyi* (Beaz-Hidalgo *et al*., 2000; Iida *et al*., 2010; Travers *et al*., 2015; Dubert *et al*., 2017). On the other hand, *Bacillus* bacteria usually attract attention because of their potential use as probiotics in aquaculture (Rengpipat *et al*., 1998; Vaseeharan & Ramasamy, 2003; Prado *et al*., 2010; Dosta *et al*., 2016; Dubert *et al*., 2017). *Bacillus* species have several attributes that are beneficial when the bacteria are fed to fish and shrimp (Michael *et al*., 2014; Newaj-Fyzul *et al*., 2014); for example, spore formation allows them to tolerate the adverse conditions of the gastrointestinal tract (acidity, enzyme action, etc.) (Ran *et al*., 2012; Dosta *et al*., 2016).

Few studies have evaluated the effects of antibiotics on bivalve defense cells and responses. In the present study, none of the immunological parameters evaluated changed after antibiotic treatment, suggesting that even with altered microbiota and the possible chemical stress caused by antibiotics, oysters maintained their immunological responses at a basal level. In fact, phagocytic capacity, a measurement of one of the most important cellular defense responses (Fournier *et al*., 2000; Allam & Raftos, 2015), was not modified, nor was cell viability, the alteration of which would indicate possible cytotoxicity of the antibiotics (Parolini *et al*., 2011; Rocha *et al*., 2014, 2015). Similarly, there was no decrease in the number of hemocytes circulating in the hemolymph; such a decrease could indicate an abrupt migration of hemocytes to tissues (Schmitt *et al*., 2012; Allam & Raftos, 2015; Carella *et al*., 2015) including the gastrointestinal tract, where alteration of the microbiota occurred. Recent studies have shown that different results can be observed depending on the antibiotic used. Trimethoprim induces an increase in the number of hemocytes in the hemolymph after seven days of exposure (Matozzo *et al*., 2015) in the clam *Ruditapes philippinarum*, while amoxicillin does not (Matozzo *et al*., 2016).

ROS assist in the destruction of pathogens, including bacteria phagocytized by hemocytes (Bachère *et al*., 2015). In the present study, 34% lower ROS production was observed in the treated group than in the control group. Lacaze *et al*. (2015) observed a decrease in ROS when studying the *in vitro* effects of three antibiotics on *Mytilus edulis* hemocytes, including erythromycin. This antibiotic was used at the same dose in the current study (100 µg mL^{-1}) and resulted in immunosuppressive effects, reducing the production of ROS and the capacity for phagocytosis of latex particles after 21 h of exposure. On one hand, the decrease in ROS could have been caused by the antibiotic treatment itself; on the other hand, it could indicate an immunosuppressive effect in the oysters caused by modification of the GTM, emphasizing the importance of the natural microbiota for the animal's defense responses (García-García *et al*., 2013). We do not exclude the possibility that the higher number of bacteria in the gastrointestinal tract of oysters could have contributed to the reduction of ROS through an antioxidant effect. Indeed, lactic acid bacteria (Amaretti *et al*., 2013), *Bacillus* spp. isolated from marine sponges (Balakrishnan *et al*., 2015) and epiphytic bacteria isolated from marine macroalgae (Horta *et al*., 2014) show considerable antioxidant and free radical scavenging activity. Horta *et al*. (2014) noted the potent antioxidant activity of two bacteria, *Shewanella* and *Vibrio*, both of which were detected in the present study. It should also be noted that these two genera contain representatives considered pathogenic to bivalves (Richards *et al*., 2008; Li *et al*., 2010; Janda & Abbott, 2014; Lokmer & Mathias-Wegner, 2015; Travers *et al*., 2015).

In conclusion, the GTM of the oyster *C. gasar* was altered by the use of a mixture of broad-spectrum antibiotics, but active oyster immune defense responses were maintained. The use of antibiotics and the standardized procedures presented herein could be applied in future studies to evaluate the role of microbiota in immunological defense after challenges with pathogenic protozoans or bacteria.

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