Journal of Invertebrate Pathology 157 (2018) 36–44

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Immune and biochemical responses in hemolymph and gills of the Patagonian freshwater mussel *Diplodon chilensis*, against two microbiological challenges: *Saccharomyces cerevisiae* and *Escherichia coli*

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ARTICLE INFO

Keywords:
Bivalves
Immune system
Cytochemical characterization
Humoral response
Oxidative stress

ABSTRACT

Immune cell characterization, immunological response and the associated gill oxidative balance were studied in the Patagonian freshwater mussel, *Diplodon chilensis,* using two microbiological immunostimulant models: *Saccharomyces cerevisiae* and *Escherichia coli.* Mussels were collected out of the breeding season in Paimún Lake and acclimated in the laboratory. Two exposure experiments were performed during two consecutive weeks: (1) mussels challenged with 500 yeast cells mL^{-1}; and (2) mussels challenged with 1000 bacteria cells mL^{-1}. Microorganisms were added in the water every two days, alternating with 6000 lyophilized cells of the green algae *Scenedesmus vacuolatus* mL^{-1}. A control group, fed with *S. vacuolatus*, was set for each treatment. Morphological cell characterization was carried out in adherent hemocytes of *D. chilensis* hemolymph under control conditions. The most important cell type observed were the hyalinocytes (representing ca. 98% of the circulating cells), agranular cells with non-central polymorphic nucleus surrounded by cytoplasm; granulocytes (cells with cytoplasmic granules and non-central rounded nucleus) represented ca. 2%. Another two cell types were occasionally detected, binucleated hyalinocytes and hemoblast-like cells but were not considered for the analyses. Both adherent hyalinocytes and granulocytes exhibit phagocytic activity towards Congo red stained yeast, which was two-fold higher in granulocytes than in hyalinocytes, regardless of the applied challenge. Total hemocyte counts were diminished in mussels challenged with *S. cerevisiae* or *E. coli*. Hydrolytic and defense cellular enzyme activities were analyzed only for hyalinocytes. Both, *S. cerevisiae* and *E. coli* increased acid phosphatase activity. *E. coli* challenge diminished hemocyte lysosomal membrane stability and increased humoral phenoloxidase activity, while *S. cerevisiae* challenge did not affect any of these variables. Mussels challenged with *E. coli* showed increased gill antioxidant response without oxidative damage, while those challenged with *S. cerevisiae* showed no change in these variables.

1. Introduction

Bivalve immunity works as a wide spectrum innate response system, which involves cellular and humoral defenses interacting in a coordinated way to recognize pathogen associated molecular patterns (PAMPs) (Loker et al., 2004; Montaño et al., 2011). PAMPs constitute structural motifs on the surface of several microorganisms, either pathogens or non-pathogens, which make them interesting models for immune response characterization in bivalve mollusks under laboratory conditions (Mar Costa et al., 2008; Husmann et al., 2011). Until now, immune response studies have been mainly focused on economically valuable marine species exposed to specific microorganisms or to isolated PAMPs, such as zymosan (β-1, 3-glucan) from *Saccharomyces cerevisiae* and lipopolysaccharides (LPSs) from Gram-negative bacteria (Aladaileh et al., 2007a; Allam and Paillard, 1998; Ciacci et al., 2009; Kuchel et al., 2010; Matozzo and Bailo, 2015). Nevertheless, the information related to the use of live bacteria and yeast cells to characterize the immune response of bivalves remains scarce.

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https://doi.org/10.1016/j.jip.2018.08.005
Received 17 November 2017; Received in revised form 2 August 2018; Accepted 7 August 2018
Available online 09 August 2018
0022-2011/

Circulating hemocytes are responsible for the cellular immune response of bivalves. Thus, morphological and histochemical characterization of circulating hemocytes based on light microscopy is needed for better recognizing the intrinsic immune capacity in different species of this group (Aladaileh et al., 2007a; Kuchel et al., 2010; Pampanin et al., 2002; Salimi et al., 2009). There is general agreement on classifying bivalve circulating cells as hyalinocytes and granulocytes, according to their well studied morphology (Hine, 1999). Among cellular immune functions, phagocytic process and lysosomal enzymes such as acid phosphatase play an important role and it has been reported that granulocytes are more phagocytically active than hyalinocytes (Kuchel et al., 2010). In addition, the proportion of the different types of circulating hemocytes can be affected by environmental changes and the total hemocyte number may be increased by exposure to PAMPs and pathogenic bacteria (Allam et al., 2000a; Barracco et al., 1999).

Lytic enzymes used to degrade phagocytized foreign particles inside the phagolysosome can be released to the extracellular space (Mohandas et al., 1985), constituting part of the humoral component of the immune response. Humoral defenses comprise constitutive or inducible biosynthesis of proteins with bacteriolytic and opsonic functions (Loker et al., 2004; Montaño et al., 2011). Invertebrate phenoloxidase (PO) is an important host defense protein involved in the synthesis of melanin, which participates in wound healing and pathogen encapsulation. To avoid undesired melanization, PO exists as an inactive form, the prophenoloxidase (proPO). In bivalve mollusks, it has been demonstrated that PAMPs from bacteria and fungi (LPS and β-1, 3-glucans) can lead the activation from proPO to PO in hemolymph reviewed by Luna-Acosta et al. (2017). Luna-González et al. (2003) reported PO activity in plasma and hemocyte lysates from adults of several marine bivalves and Hellio et al. (2007) reported much higher PO levels in plasma than in hemocytes of *Crassostrea gigas*.

Experiments involving feeding fish and shrimps with *S. cerevisiae* cells or derived β-glucans suggest that yeast have wide potential for improving the health status of organisms in aquaculture procedures (Meena et al., 2013). Positive results on the immune response of humans and other vertebrate species have also been reported after feeding with or intra peritoneal administration of yeast β-glucans (Li et al., 2014; Volman et al., 2008). In addition, antioxidant activity of yeast cell wall components, such as aromatic side chains and thiol groups of proteins, has been detected and studied for potential use of yeast as animal food (Jaehrig et al., 2008). As for the use of bacteria for immunological challenge, it has been shown that LPSs constitute the major endotoxin involved in the pathogenesis of *Escherichia coli,* causing inflammation and oxidative stress in mammals (Kheir-Eldin et al., 2001). Bivalves exposed to bacterial stimuli show increased immune response (Bianchi et al., 2015; Husmann et al., 2011), although these bacteria potentially can be harmful for the organism, causing oxidative stress and cellular damage (Bianchi et al., 2015; Sabatini et al., 2011). *In vitro* experiments using live *E. coli* have been performed to evaluate surface interactions between bacteria and *Mytilus galloprovincialis* hemocytes (Canesi et al., 2001). In addition, *E. coli* has been used as positive control to evaluate the effects of different *Vibrio* species on *M. galloprovincialis* hemolymph bactericidal activity in *in vivo* experiments (Ciacci et al., 2009).

Bivalves remove and accumulate microorganisms from the water column and from the microbenthos via their filter-feeding activity (Lee and Silk, 2013). During this process, the gills and the hemolymph beneath the gill surface may be continuously challenged by microbial toxic compounds, which could lead to increased production of oxygen reactive species (ROS). This increase may be associated with enhanced cellular immune response and/or to an oxidative stress condition (Almeida et al., 2007; Husmann et al., 2011). Enzymatic antioxidant defenses such as catalase (CAT) neutralize the cellular ROS increase, while glutathione-S-transferase (GST) detoxifies the cell from toxic metabolites such as LPSs (Almeida et al., 2007; Revathy et al., 2012). However, when this defense system is overwhelmed, oxidative stress may cause membrane destabilization in hemocyte lysosomes (Viarengo et al., 2007) and peroxidation of cell membrane phospholipids, which could lead to further oxidative damage to cell components (Aldini et al., 2007; Mattie and Freedman, 2001).

Although freshwater mussels have little economic value, their ecological importance is reflected by their enormous contribution to the maintenance and preservation of oligotrophic water bodies, e.g. (Rocchetta et al., 2014; Soto and Mena, 1999). *Diplodon chilensis* (Gray 1828) is a native freshwater mussel from Patagonian lakes and rivers of Argentina and Chile. This mussel can feed on and digest coliform bacteria (Lara et al., 2002), suffering moderate oxidative stress effects (Bianchi et al., 2015; Sabatini et al., 2011). Some immune and antioxidant responses have been studied previously as biomarkers in *D. chilensis* that were chronically exposed to sewage polluted water (Bianchi et al., 2014a, 2014b) and to *E. coli* in the laboratory (Bianchi et al., 2015; Sabatini et al., 2011). Bianchi et al. (2015) observed that *D. chilensis* fed with a β-glucan rich diet (*Euglena gracilis* cells) display differential immune and antioxidant responses compared with mussels fed with *E. coli*. In addition, Castro et al. (2017) characterized the two principal cell types of this species and studied the modulation of the immune response against *E. coli* by the insecticide azinphos methyl. This work aims to further characterize the immune cells of *D. chilensis* and to compare the immune response and the associated antioxidant response in gills against two microbiological challenge models, *S. cerevisiae* and *E. coli*.

2. Materials and methods

2.1. Microorganisms

Commercially available fresh *S. cerevisiae* cells were suspended in sterile tap water and centrifuged at 500 × *g* for 15 min in order to obtain washed cells. Cell density was determined by direct counting in a Neubauer cell counting chamber, using a light microscope at 400× and then diluted to obtain an appropriate working cell suspension.

E. coli JM 109 strain was donated by the Department of Biological Chemistry, Faculty of Exact and Natural Sciences, University of Buenos Aires (FCEN, UBA) and used to prepare a suspension of 1.5×10^8 cells mL^{-1} in sterile saline solution (NaCl 0.9%, Merck) according to Castro et al. (2017). This suspension was then diluted to obtain an appropriate working cell suspension.

Scenedesmus vacuolatus (Chlorophyceae, Chlorophyta) BAFC CA4 strain was provided by Culture Collection of the Laboratory of Phycology, Department of Biodiversity and Experimental Biology, FCEN, UBA. Culture conditions were based on Sabatini et al. (2009). After 25 days, cells were lyophilized and stored at −20 °C.

2.2. Diplodon chilensis

Adult individuals (n = 74; 67.60 ± 0.38 mm total shell length) were obtained by scuba diving (1–2 m depth), from Paimún Lake (39°44.78′S 71°37.48′W), which has no fecal bacteria and less than 10 MPN/100 mL total coliform bacteria (Castro et al., unpub.) in Lanin National Park in March-April 2013, in order to avoid the mussels' reproductive season (Peredo and Parada, 1986). Based on Rocchetta et al. (2014), the estimated age of mussels was between 30 and 40 years. Bivalves were transported to the laboratory in plastic containers with water and ice packs. The collected mussels were acclimated for 10 days in 1.5 L glass flask (previously sterilized, two individuals each), containing 1 L of aerated dechlorinated tap water at 15 ± 1 °C. During this period, mussels in each flask were fed with 6000 lyophilized cells of *S. vacuolatus* mL^{-1} (final concentration) every two days. Water was changed before each feeding. Mussels were fasted for 48 h before starting the experiments (Castro et al., 2017).

2.3. Experimental design

Microbiological exposure was performed in two separate experiments carried out in consecutive weeks. In the first experiment, treated mussels received 500 yeast cells mL^{-1}. In the second experiment, mussels were exposed to a final concentration of 1000 bacteria cells mL^{-1}. Both microorganisms were added to the water every two days, alternating with 6000 lyophilized cells of *S. vacuolatus* mL^{-1}, for one week. Control groups were set for each experiment, in which mussels were fed with algae every two days, for one week. In all cases, water was changed before adding the microorganisms. Temperature was maintained at 15 ± 1 °C.

2.4. Sample processing

According to Castro et al. (2017), two hematological variables were studied each day of experimentation over three consecutive days. Six mussels (two per flask) from the treated group and the corresponding control were exposed to the microbiological challenge and then processed in the same order. The results from the two individuals in each flask were averaged to obtain an individual data point (n = 3 for each treatment/variable). In addition, one gill pair from each flask was sampled to study oxidative balance (n = 9 for each treatment). Individuals were placed on ice for 5 min, and then two mL of hemolymph were withdrawn from the anterior adductor muscle for immediate immunological analysis and aliquoted into cold sterile microcentrifuge tubes containing anticoagulant solution (3 g L^{-1} glucose and 0.36 g L^{-1} trisodium citrate, 60 mOsm L^{-1}, pH 7). Next, mussels were opened and gills were excised, weighed and homogenized (20,000 rpm) in cold 100 mmol L^{-1} sodium phosphate buffer (PB), pH 7.0, 1:5 w/v, with protease inhibitor (0.2 mmol L^{-1} phenylmethylsulphonyl fluoride, PMSF, Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged (15 min at 11,000 × *g* at 4 °C), and supernatants were saved for the analysis.

2.5. Immune cell characterization

Cell morphological analysis was carried out in the hemolymph of five mussels from the control group using the open access software ImageJ 1.51w. Fresh hemolymph (30 μL) was smeared onto microscope slides and allowed to adhere for 20 min in a wet chamber at room temperature. The adhered cells were fixed with ethanol 96% for 1 min, stained with Wright solution (Biopack, Argentina) for 5 min and then counterstained with Giemsa solution (Biopack, Argentina) for 15 min. Slides were washed with distilled water, dried for 24 h at room temperature and mounted in DPX (Sigma). Approximately 45 cells per slide (five slides per mussel) were identified and photographed under light microscopy at (150–1500 ×). According to Kuchel et al. (2010), hyalinocytes were identified as agranular cells, with round or irregular nucleus. Granulocytes were described as cells rich in cytoplasmic granules, with round nucleus. The morphometric parameters cellular and nuclear diameter and nuclear circularity (NuCirc.) were measured in order to calculate the nucleus/cytoplasm ratio (N/C ratio). Briefly, working with calibrated photos (pixel/μm), maximum nuclear and cellular (Feret's diameter) were calculated as the longest distance between any two points along the boundary. NuCirc. was defined as 4π (Area/ [Perimeter]^2), where a value = 1 means a perfect circle. N/C ratio was calculated only for cells having a NuCirc. = 0.85, which were the most abundant among both hyalinocytes and granulocytes (https://imagej.nih.gov/ij/).

2.6. Immune response

2.6.1. Total number of circulating hemocytes

The total number of cells for each individual was counted 4 × in a Neubauer chamber and averaged. The results from the two individuals in each flask were then averaged to obtain an individual data point (n = 3) (Castro et al. 2017).

2.6.2. Hemocyte population

Slides were prepared as described in Section 2.5 but, after washing with distilled water, two slides per mussel were immediately observed under light microscopy (150–1500 ×). A total of 300 cells were identified from the two slides and the percentage of cells of each type with respect to the total number of hemocytes observed was estimated.

2.6.3. Phagocytosis

Phagocytic activity was measured according to Castro et al. (2017) with some modifications. 100 μL of hemolymph were mixed with Congo red (Fluka) stained yeast suspension (twice the number of cells with respect to the number of viable hemocytes; see Section 3.3), in sterile anticoagulant solution) and incubated at room temperature for 30 min. 50 μL of mixture were smeared onto clean microscope slides and cells were allowed to adhere for 20 min in a wet chamber at room temperature. Smears were then fixed with 96% ethanol for 1 min, stained with Giemsa solution, and washed and mounted with distilled water. Cells were counted in duplicate (minimum of 300) under light microscopy (150–600 ×). Phagocytic activity was calculated as yeast cells phagocytized by hyalinocytes or granulocytes/total number of adherent cells of each type.

2.6.4. Cellular enzyme activity

Cellular enzyme activity was measured according to Castro et al. (2017) with some modifications. Fresh hemolymph (50 μL) samples were smeared onto clean microscope slides, and cells were allowed to adhere for 20 min in a wet chamber at room temperature. The adhered cells were then fixed with 96% ethanol for 1 min and processed for acid and alkaline phosphatases with naphthol AS-BI phosphate (Sigma) as substrate (Cima et al., 2001), at pH = 5.02 and 9 for acid and alkaline phosphatase, respectively.

2.6.5. Phenoloxidase activity

Phenoloxidase activity was measured according to Bianchi et al. (2014a). Cell-free supernatant was obtained by centrifugation at 500 × *g* for 20 min at room temperature, and was then incubated with L-DOPA (Sigma) in PBS, 3 mg mL^{-1} for 4 h. After incubation, changes in absorbance at 490 nm were recorded for 20 min. Negative and spontaneous substrate oxidation controls were performed. One arbitrary unit of activity (U PO) was defined as a 0.001 change in absorbance. Results were referred to mg of proteins.

2.7. Cytotoxicity

Cytotoxic effects were analyzed in fresh hemolymph samples. Hemocyte viability and lysosomal membrane stability were studied using the trypan blue exclusion assay and neutral red retention time 50% (NRRT 50), respectively (Bianchi et al., 2014a; Castro et al., 2017).

2.8. Gill oxidative balance

GST activity was measured according to Habig et al. (1974); changes in absorbance at 340 nm were recorded for 5 min in the following mixture: 10 μL of homogenate supernatant, 5 μL of reduced glutathione (Sigma, 100 mmol L^{-1} GSH in PB) and 5 μL of 1 chloro-2,4 dinitrobenzene (100 mmol L^{-1} CDNB, Sigma, in ethanol) at a final volume of 1 mL in PBS 100 mmol L^{-1}, pH 6.5. One unit of activity was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB per min, at 25 °C.

CAT activity was assessed according to Aebi (1984), changes in absorbance at 240 nm were recorded for 30 s in the following mixture: 80 μL of homogenate supernatant and, 40 μL of H_2O_2 10 mmol L^{-1}, at a final volume of 2 mL in PBS 50 mmol L^{-1}, pH 7. One unit of activity

was defined as the amount of enzyme needed to catalyze the transformation of 1 mmol of H_2O_2 per min, at 25 °C.

Damage to lipids was estimated by the thiobarbituric acid reactive substances method (TBARS) according to Fraga et al. (1988). Homogenate supernatant was mixed with thiobarbituric acid (TBA, Sigma) solution and incubated at 95–100 °C for 15 min. The mixture was then cooled at room temperature and centrifuged for 15 min at 11,000 × *g*. Changes in absorbance were read at 535 nm and TBARS concentration was estimated using an extinction coefficient of 156 mM^{-1} cm^{-1}. Oxidative balance results were referred to mg of proteins. Protein concentrations in hemolymph and gill tissue were measured by the Bradford's method (1976).

2.9. Statistical analysis

Data are presented as mean ± standard error (SEM). Normality and homogeneity of variance were checked by Kolmogorov-Smirnoff and Levene tests, respectively. Data were transformed by square root arcsine or Log10 when needed. All data, except those of phagocytic activity were tested by two-tail Student's test. Differences between spreading and round cells on cellular diameter, nuclear circularity and N/C ratio were tested by two-tail paired Student's test. Phagocytic activity was analyzed by two-way ANOVA and Tukey *post hoc* comparisons. Significant differences were assumed when $p < 0.05$.

3. Results

3.1. Immune cell characterization

Two main cell types were observed in *D. chilensis* hemolymph: hyalinocytes (ca. 98% of total adherent hemocytes) and granulocytes (ca. 2%) (Fig. 1). Other cell types, which were occasionally observed, were identified as hemoblast-like cells (Aladaileh et al., 2007a) and binucleated cells (Pampanin et al., 2002) (Figs. 1B and 2A and B, respectively). Hyalinocytes appeared as agranular cells with a basophilic polymorphic nucleus surrounded by acidophilic or basophilic cytoplasm (Fig. 1). These cells showed pseudopods of varying lengths or thin projections recognized as filopods (Fig. 2C). Granulocytes had a basophilic, non-central, ovoid nucleus surrounded by numerous cytoplasmic granules and did not show important cellular projections (Fig. 1). Due to the low percentage of granulocytes, the detailed morphometric characterization was only carried out for hyalinocytes. In these cells, the most frequent cellular diameter class was 15–21 µm (76%). Within this interval, the most frequent nuclei diameter and circularity were 7–9 µm (59%) and 0.75–0.85 (56%), respectively. Hyalinocytes were observed in two shapes: spreading or round (Fig. 1). Spreading hyalinocytes were less frequent and larger than round hyalinocytes; spreading cells nuclei showed lower circularity than those of round cells (Table 1). The N/C ratio was not related to the cellular type and showed no significant differences between spreading and round cells (Table 1). Binucleated cells appeared as hemocytes without granules, with irregular nuclei, and with or without short filopods (Fig. 2B and A, respectively); while hemoblast-like cells appeared as hyalinocytes with a high N/C ratio (Fig. 1B).

3.2. Immune response

3.2.1. Total number of circulating hemocytes

The total circulating hemocyte count significantly decreased in both challenged groups compared with their respective controls. The level of this variable was reduced by 24.75% ($p < 0.01$) in the *S. cerevisiae* group and by 41.68% ($p < 0.05$) in the *E. coli* group (Fig. 3).

3.2.2. Hemocyte population

The percentage of adherent hyalinocytes was not affected by *S. cerevisiae* or *E. coli* challenge. In the *S. cerevisiae* challenge the values obtained were 97.13 ± 0.35% *vs.* 93.70 ± 1.31%, n = 3, for control and treated groups respectively. In the *E. coli* challenge the percentage of adherent hyalinocytes were 87.06 ± 2.15% *vs.* 85.08 ± 2.89%, n = 3, for control and treated groups respectively. No significant differences were observed in the percentage of adherent granulocytes in the *S. cerevisiae* challenge (2.88 ± 0.55% *vs.* 1.60 ± 0.40%, for control and treated groups respectively, n = 3), nor for *E. coli* challenge (8.65 ± 2.35% *vs.* 12.03 ± 3.11%, for control and treated groups respectively, n = 3). Binucleated and hemoblast-like cells were scarce, and were not included in the statistical analysis.

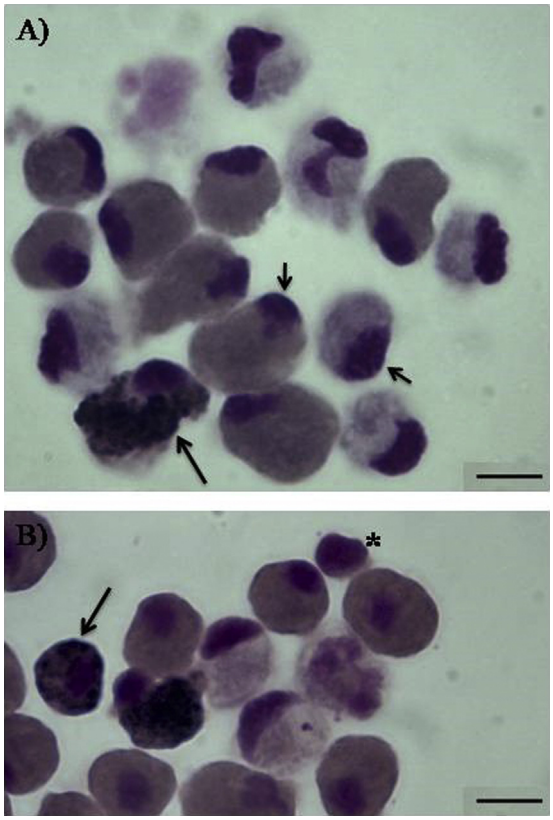

Fig. 1. Circulating hemocytes in *Diplodon chilensis* hemolymph stained with Wright–Giemsa method: spreading and round hyalinocytes showing nucleus shape variability are indicated by short arrows; spreading and round granulocytes are indicated by long arrows. A hemoblast-like cell is indicated by an asterisk. Scale bar = 10 µM.

3.2.3. Phagocytosis

Both adherent hyalinocytes and granulocytes of *D. chilensis* ingested Congo red stained yeast cells. Phagocytic activity of granulocytes was two-fold higher than that of hyalinocytes ($p < 0.001$) when mussels were challenged with either *S. cerevisiae* or *E. coli*. Challenge effect on this variable was only observed in granulocytes of mussels exposed to *E. coli* with a significant reduction in challenged mussels with respect to control ($p < 0.01$). No challenge effect was observed in adherent hyalinocytes (Fig. 4).

3.2.4. Cellular enzyme activity

Due to the small number of adherent granulocytes observed, only adherent hyalinocytes were considered for data analysis. Acid phosphatase activity increased significantly in both challenged groups with respect to their corresponding controls. This variable was augmented 8-fold ($p < 0.001$) upon *S. cerevisiae* challenge and 27-fold ($p < 0.001$) upon *E. coli* challenge (Fig. 5A, B). Alkaline phosphatase activity was not significantly affected by *S. cerevisiae* challenge (5.36 ± 2.56% *vs.* 3.31 ± 2.51%, for control and the challenged group, respectively,

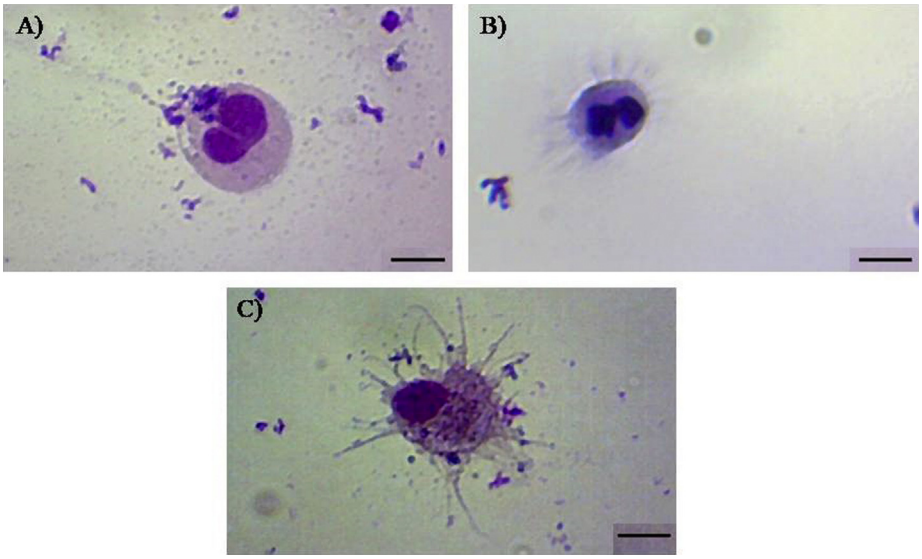

Fig. 2. Hemolymph smears stained with Wright-Giemsa in *Diplodon chilensis*. Binucleated hyalinocytes (A and B) and circulating hyalinocyte showing filopods with different lengths (C). Scale bar = 10 μM.

Table 1
Morphological characterization of *Diplodon chilensis* circulating hyalinocytes. Values are mean ± standard error, n = 5. Asterisks denote significant differences between groups (Two-tail paired Student's test, $^{***}p < 0.001$).

Parameter	Spreading	Round
Percentage	36.32 ± 4.734	63.68 ± 4.734
Cellular diameter (μm)	19.42 ± 0.148***	17.41 ± 0.127
NuCirc.	0.69 ± 0.008	0.73 ± 0.006***
N/C ratio	0.48 ± 0.006	0.49 ± 0.005

n = 3), or *E. coli* challenge (5.64 ± 4.96% *vs*. 29.59 ± 12.27% for the control and challenged group, respectively, n = 3).

3.2.5. Phenoloxidase activity

Phenoloxidase activity showed no significant differences in cell-free supernatant from *D. chilensis* challenged with *S. cerevisiae* compared to the control group (0.35 ± 0.04 *vs.* 0.26 ± 0.02, respectively, n = 3). In contrast, this variable was about 3.4-fold increased by *E. coli* challenge ($p < 0.001$) (Fig. 6). Protein concentration did not vary among groups (data not shown).

3.3. Cytotoxicity

There were no significant differences in cell viability between challenged and control groups (97.98 ± 0.57% *vs.* 97.81 ± 0.48%, n = 3 for *S. cerevisiae,* and 97.63 ± 0.48% *vs.* 95.33 ± 1.90%, n = 3 for *E. coli*, respectively). Lysosomal membrane stability, evaluated as NRRT50, was not significantly affected by *S. cerevisiae* challenge (24.06 ± 0.79 min *vs.* 24.95 ± 0.74 min, n = 3 for control and treated group, respectively); but was significantly diminished by 35% after *E. coli* challenge ($p < 0.001$, Fig. 7).

3.4. Gill oxidative balance

After *S. cerevisiae* challenge, gill GST and CAT activities, and TBARS levels showed no difference between treated and control groups (0.023 ± 0.005 *vs.* 0.017 ± 0.002, n = 5 for GST; 2.59 ± 0.11 *vs.* 2.50 ± 0.035, n = 5 for CAT; 1.19 ± 0.15 *vs.* 1.12 ± 0.04, n = 4 for TBARS levels). In contrast, gill GST and CAT activities were increased about 2-fold and 4-fold, respectively ($p < 0.01$) in gills of *E. coli*-challenged mussels with respect to the control (Fig. 8A, B). TBARS levels decreased significantly ($p < 0.05$) after *E. coli* challenge (Fig. 8C).

4. Discussion

Our work characterizes the immune cells of the freshwater mussel *Diplodon chilensis* and its hemato-immunological response to challenge with the yeast *S. cerevisiae* or with the fecal bacterium *E. coli*. The microorganisms were applied as food items, which is not common in previous research, but is ecologically relevant and avoids animal manipulation during the exposure period. The microbial concentrations were selected in order to elicit both an immune response and oxidative stress in gills of *D. chilensis*, based on results from previous studies (Bianchi et al., 2015; Sabatini et al., 2011). Due to size differences between *S. cerevisiae* and *E. coli* cells, physiological responses were

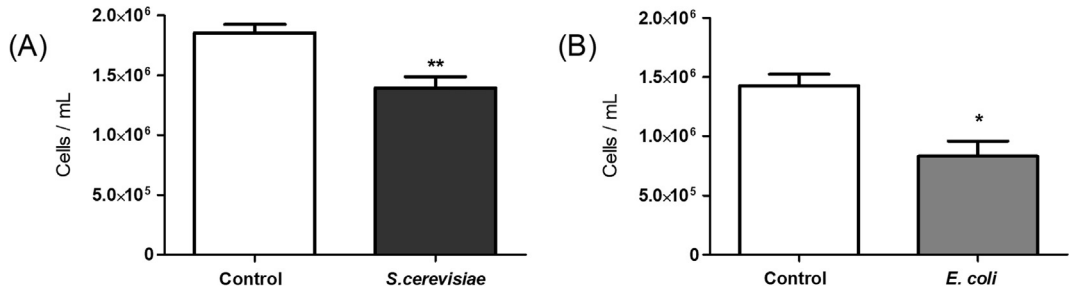

Fig. 3. Total number of circulating hemocytes (Cell/mL) in hemolymph of *Diplodon chilensis* challenged with *Saccharomyces cerevisiae* (A) or *Escherichia coli* (B) (mean ± standard error; n = 3). Asterisks denote significant differences between groups (Two-tail Student's test, $^{**}p < 0.01$ and $^{*}p < 0.05$).

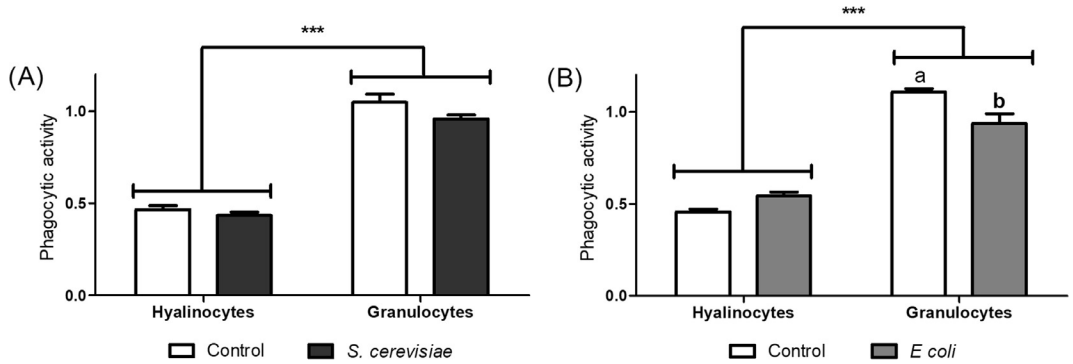

Fig. 4. Phagocytic activity in circulating hemocytes (Congo red stained yeast cells engulfed by hyalinocytes or granulocytes/total number of cells of each type) of *Diplodon chilensis* challenged with *Saccharomyces cerevisiae* (A) or *Escherichia coli* (B) (mean ± standard error; n = 3). Different characters denote significant differences between challenged and control groups (Tukey, $p < 0.01$). *** on the horizontal line denote $p < 0.001$ between hyalinocytes and granulocytes (Two-way ANOVA).

compared between the two challenges only qualitatively. We performed the cellular characterization, phagocytosis and phosphatase activity studies on adhered cells, therefore, the results of these assays are limited to adherent cells and may not reflect the total hemocytes. For example, some cells phagocytizing many yeast cells could have less adhesion capacity, thus leading to the underestimation of phagocytic activity. However, the results obtained in this work broaden knowledge about the immune response of freshwater bivalves and highlight the differential immune and antioxidant responses displayed by mussels upon exposure to these two microbiological challenges.

4.1. Immune cell characterization

Morphological characterization of hemocytes is a basic tool for studies aimed to elucidate the causes of immune susceptibility or death in bivalves (Fisher, 1985). Contrarily to the well studied hemolymph cell types of marine bivalves (Ciacci et al., 2009; Kuchel et al., 2010), investigations of hemocyte types in freshwater bivalves are scarce. A morphological and cell size-based hemocyte classification was previously published for *Anodonta cygnea* (Salimi et al., 2009). More recently, Evariste et al. (2016) characterized the *Dreissena polymorpha* hemocytes during the gametogenesis period, and Castro et al. (2017) conducted a cytological study of *D. chilensis* exposed to the organophosphorus pesticide azinphos-methyl. In accordance with previous studies in bivalves, circulating hemocytes of *D. chilensis* can be classified into two major groups: hyalinocytes and granulocytes (Aladaileh et al., 2007a; Castro et al., 2017; Evariste et al., 2016; Hine, 1999; Husmann et al., 2011; Kuchel et al., 2010; Pampanin et al., 2002; Salimi et al., 2009). In *D. chilensis,* more than 90% of the circulating cells correspond to hyalinocytes (Castro et al., 2017; this study). Similarly, the main cell type observed in *Dreissena polymorpha* was also characterized as hyalinocyte (Evariste et al., 2016). This predominance of hyalinocytes over granulocytes also has been observed for marine bivalves such as the scallop *Nodipecten subnodosus* (Estrada et al., 2013), the venus clam *Chamelea gallina* (Pampanin et al., 2002) and the oyster *Saccostrea glomerata* (Aladaileh et al., 2007a). In contrast, granulocytes are the most abundant in the smooth venus clam *Callista chione* (Matozzo and Bailo, 2015) and the oyster *Pinctada imbricata* (Kuchel et al., 2010). In *D. chilensis*, hyalinocytes appear as spreading or round cells, the former having slightly larger size and lower nuclear circularity. Because no other important differences between the two shapes are evident,; it is likely that both shape classes represent higher or lower adherence to the slide rather than two different cell types. Evariste et al. (2016) proposed that cell size could be affected by the fixation protocol used for staining, and that the characterization of different cell types based on cell size is not sufficient for light microscopy analysis due to cell plasticity. As well as in other bivalve species, binucleated hyalinocytes (e.g. Pampanin et al., 2002) and hemoblast-like cells, (e.g. Aladaileh et al., 2007a, Evariste et al., 2016) are observed in *D. chilensis* hemolymph. It has been suggested that the presence of binucleated hyalinocytes indicates that these cells may retain the ability to divide (Pampanin et al., 2002) and, while hemoblast-like cells would not contribute to defensive responses, e.g. phagocytosis, they probably act as prohaemocytes (Cima et al., 2001; Evariste et al., 2016).

4.2. Immune response

Total hemocyte number has been shown to increase in hemolymph of marine bivalves exposed to microbiological stimuli, e.g. Oubella et al. (1994), Allam and Paillard. (1998), and Allam et al. (2000b) found increased numbers of circulating hemocytes in the clam *Ruditapes philippinarum* injected with the pathogen *Vibrio tapetis* (Vibrio P1). In contrast, the number of circulating hemocytes diminishes with respect to the control in *D. chilensis* challenged with *S. cerevisiae* or *E. coli*

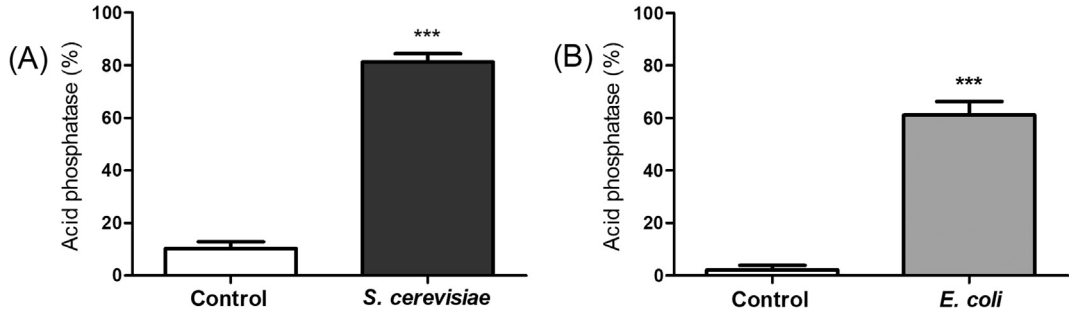

Fig. 5. Acid and alkaline phosphatase activity (as percentage of positive cells with red reaction) in circulating hyalinocytes of *Diplodon chilensis* challenged with *Saccharomyces cerevisiae* (A, C) or *Escherichia coli* (B, D) (mean ± standard error; n = 3). Asterisks denote significant differences between groups (Two-tail Student's test, *** $p < 0.001$).

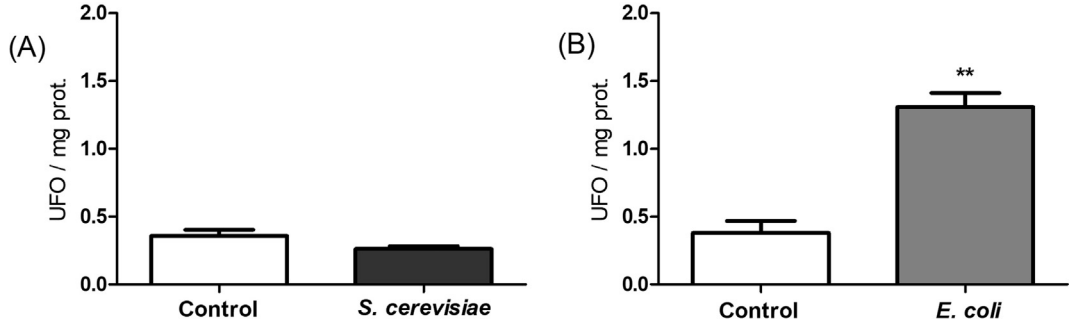

Fig. 6. Phenoloxidase activity in arbitrary units per mg of proteins (UPO / mg prot.) in hemolymph cell-free supernatant from *Diplodon chilensis* challenged with *Saccharomyces cerevisiae* (A) or *Escherichia coli* (B) (mean ± standard error; n = 3). Asterisks denote significant differences between groups (Two-tail Student's test, $^{**}p < 0.01$, $^{***}p < 0.001$).

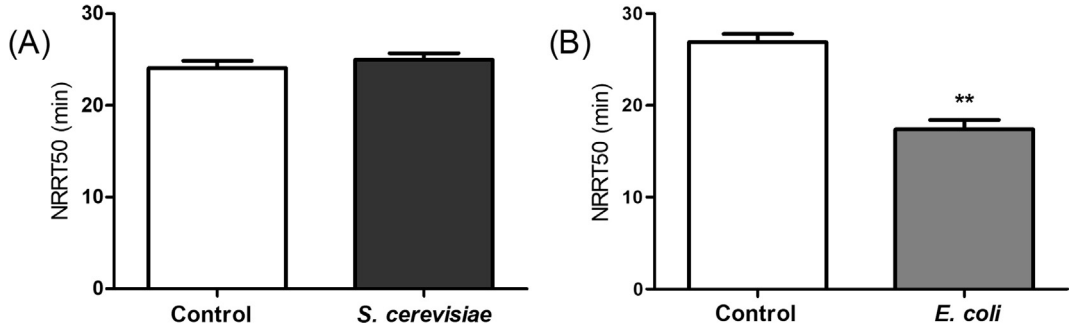

Fig. 7. Neutral Red Retention Time 50% in circulating hemocytes of *Diplodon chilensis* challenged with *Saccharomyces cerevisiae* (A) or *Escherichia coli* (B) (mean ± standard error; n = 3). Asterisks denote significant differences between groups (Two-tail Student's test, $^{**}p < 0.001$).

suspended in water. This effect could be related to the way in which the challenge was applied, because *D. chilensis* hemocytes can migrate from hemolymph to tissues that are in direct contact with the environment, such as gills and mantle, as a response to the presence of microorganisms in water (Bianchi et al., 2015). We obtained different hemocytes counts between the controls for the *E. coli* and *S. cerevisiae* challenges. This difference could be related to the different times of experimentation in the laboratory; the *E. coli* challenge was performed one week later than the *S. cerevisiae* challenge.

Phagocytosis is one of the main defense mechanisms in invertebrate immune systems (reviewed by Donaghy et al. (2009)). In *D. chilensis*, granulocytes are scarce compared to hyalinocytes but are twice as active in terms of phagocytosis. These results are in accordance with those obtained by Kuchel et al. (2010) for *P. imbricata* and by Aladaileh et al. (2007a) for *S. glomerata*. In addition, Terahara et al. (2006) reported that hyalinocytes from *C. gigas* are more efficient for engulfing latex beads, while granulocytes preferentially phagocytose bacteria and yeast. However, hyalinocytes represent about 90% of *D. chilensis* adherent hemocytes, and, thus, they appear to account for most of the phagocytic response. Because granulocytes are highly phagocytic, a particular role of these cells in localized areas cannot be dismissed.

According to their phagocytic capacity, *D. chilensis* hyalinocytes show lysosomal enzyme activity related to phagocytic processes such as acid phosphatase (Pampanin et al., 2002). The presence and localization of lysosomal enzymes were studied for several marine bivalves e.g. (Estrada et al., 2013; Matozzo and Bailo, 2015; Pampanin et al., 2002). Acid phosphatase is considered to be a typical lysosome marker related to the immunological state of the individual, and may participate in intracellular digestion of proteins, carbohydrates and lipids (Cheng, 1986). In addition, it was reported that acid phosphatase is also frequently found in extralysosomal areas and in different cell types (Borgers and Verheyen, 1985). Hyalinocyte acid phosphatase activity increases in *D. chilensis* challenged with both *S. cerevisiae* and *E. coli*; however, no changes in phagocytic activity are observed in these cells. This suggests that acid phosphatase is not specifically related to lysosomal function. Alternatively, it can be speculated that the capacity of *D. chilensis* hyalinocytes to engulf yeast cells is constitutively high and the induction of enzyme activity enhances the degradation of phagocytized particles. In turn, alkaline phosphatase is widely distributed among different groups, playing an important role in cell phosphate metabolism (Xiao et al., 2002) and immune response (Lallès, 2013; Soudant et al., 2013). In mollusks, this enzyme has been used as biomarker of stress response against metal exposure (Suresh et al., 1993). In this work, no statistically significant changes in alkaline phosphatase activity were observed in hyalinocytes from *D. chilensis* exposed to *E. coli*. The similarity in the response of acid phosphatase to both *S. cerevisiae* and *E. coli* suggests that receptors with affinity for both LPS and β-glucans, such as the β-glucan binding protein (β-GBP) (Jayaraj et al., 2008), could be regulating acid phosphatase activity in *D. chilensis* hyalinocytes. On the other hand, the lack of significant activation obtained in this study suggests that alkaline phosphatase activity is not regulated by this kind of receptor.

Activation of ProPO to PO after PAMPs recognition, both in plasma and in hemocytes, has been interpreted as evidence of the participation of PO in the immune defense of bivalves (Aladaileh, et al 2007b; Hellio et al. 2007; Luna-Gonzáles et al. 2003). In our study, *D. chilensis* PO activity was stimulated by exposure to *E. coli* but was not affected by *S. cerevisiae*. On one hand, these results suggest that PO acts as a humoral defense against bacterial infection, similar to that in marine bivalves (reviewed by Luna-Acosta 2017). On the other hand, these results suggest that PO activity could be regulated by a cell signaling pathway that includes a receptor sensitive to LPS but not to β-1, 3 glucans. In contrast, Jayaraj et al. (2008) have reported that β-GBP may recognize both bacterial and yeast PAMPs, triggering agglutination processes and prophenoloxidase activation in *Perna viridis*. Therefore, in contrast to the marine bivalves, phenoloxidase activity of *D. chilensis* does not appear to be regulated by β-GBP-like receptors. Thus, the positive modulation by LPSs should be mediated by other receptors. Taken

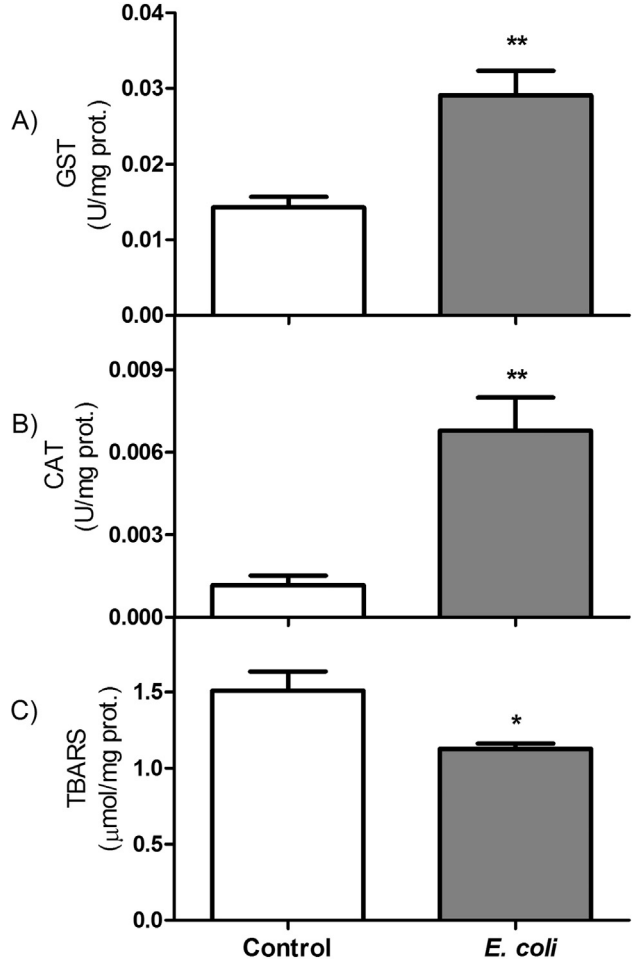

Fig. 8. Glutation-S-transferase activity (GST) (A), catalase activity (CAT) (B) and lipid peroxidation (TBARS) (C) in gill tissue from *Diplodon chilensis* challenged with *Escherichia coli* (mean ± standard error). Asterisks denote significant differences between groups (Two-tail Student's test, $^{**}p < 0.01$, n = 5 and n = 4 for GST and CAT, respectively; $^{*}p < 0.05$, n = 5 for TBARS,).

together, the effects of *E. coli* and *S. cerevisiae* on acid phosphatase and PO suggest the existence of a receptor sensitive to both LPS and β-1, 3 glucans, for example, β-GBP and a different receptor that is particularly sensitive to LPS in *D. chilensis*. In this regard, Wang et al. (2015) reported that the phagocytic receptor of *C. gigas*, cgNimc, binds to Gram negative bacteria but not to Gram positive bacteria or fungi. However, the nature of the pattern recognition receptors has not been identified in freshwater bivalves.

4.3. Cytotoxicity

Immuno-stimulant components isolated from yeast (β-glucan) and bacteria (e.g. *Vibrio* vaccine, *Listonella* antigen) may cause either cytotoxic effects on hemocytes after a certain threshold concentration (Hauton and Smith, 2004) or favor protection mechanisms against harmful microorganisms. For example, LPSs enhance the defense of *Ruditapes decussatus* against live *Vibrio splendidus* (Prado-Alvarez et al., 2012). However, as pointed out by Jaehrig et al. (2008) and Prado-Alvarez et al. (2012), the use of whole cells could produce different results. Prado-Alvarez et al. (2012) found that mussels exposed to LPSs show low levels of damaged hemocytes; while those exposed to dead cells of *Vibrio splendidus* show reduced cell viability. In this regard, feeding with β-glucan (paramylon)-rich *E. gracilis* cells enhances hemocyte protection against *E. coli* in *D. chilensis* (Bianchi et al., 2015). In this work, hemocyte viability was not significantly affected by yeast nor by bacteria; however, NRRT50 showed that exposure to *E. coli* reduced the lysosomal membrane stability of *D. chilensis* hemocytes, while exposure to *S. cerevisiae* had no effect on this variable. It could be argued that oxidative stress caused by bacteria, which was previously detected in *D. chilensis* (Bianchi et al., 2015; Sabatini et al., 2011), may be the cause of lysosomal membrane destabilization we observed (Viarengo et al., 2007). Hauton and Smith (2004) reported that in the lobster *Homarus gammarus*, lysosomal membranes are destabilized by low doses of yeast β-glucan. The lack of cytotoxicity of yeast β-glucan on hemocytes of *D. chilensis* could be explained by interspecific differences but could also be result of the high amount of antioxidant components present in the yeast cells, which would compensate any prooxidant activity of β-glucan (Jaehrig et al., 2008). This would make whole yeast cells suitable and harmless immunostimulants.

4.4. Gill oxidative balance

Gills possess large surfaces areas in direct contact with the environment and are, therefore, more prone to oxidative damage than other organs (Almeida et al., 2005). Individuals of *D. chilensis* exposed to *E. coli* showed increased activity of gill enzymatic defenses (GST, CAT), as was observed for this species in previous work using half the amount of bacteria as in our study (Bianchi et al., 2015). These results are in agreement with those published by Umasuthan et al. (2012) and Revathy et al. (2012), where GST expression is increased after bacteria and LPSs challenge in gills of two species of bivalves. This response, together with the increased CAT activity, seems to be effective to avoid oxidative damage to lipids (TBARS). In contrast, *S. cerevisiae* causes no changes to gill oxidative balance in these mussels, further supporting the idea that *S. cerevisiae* stimulates *D. chilensis* immune system but with no harmful associated effects.

5. Conclusions

The two main cells types with phagocytic capacity in *D. chilensis* hemolymph are hyalinocytes (the most abundant) and granulocytes (the most phagocytic); in addition, binucleated and hemoblast-like cells were observed. The presence of *Saccharomyces cerevisiae* and *Escherichia coli* stimulates acid phosphatase similarly in the hyalinocytes. Humoral phenoloxidase activity is stimulated only by the bacteria, suggesting the presence of β-GBP-like receptors (responsive to both kinds of challenge) and other receptors particularly sensitive to Gram negative bacteria.

Acknowledgments

This study was supported by grants CONICET PIP11220130100529CO to CML and ANPCYT PICT 2013-1415 to AV, with permission N° 815 from the National Parks Administration. We would like to thank material support from Centro de Ecología Aplicada del Neuquén, Cooperativa de Agua Potable y Saneamiento de San Martín de los Andes and Escuela de Buceo San Martín de los Andes.

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