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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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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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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).