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Is pallial mucus involved in *Ostrea edulis* defenses against the parasite *Bonamia ostreae*?

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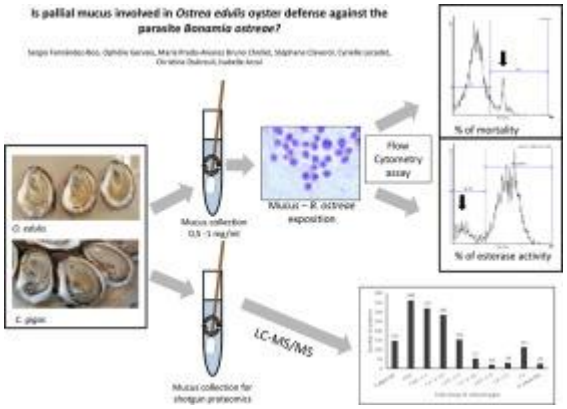
Abstract :

Bonamia ostreae is an intrahemocytic parasite that has been responsible for severe mortalities in the flat oyster *Ostrea edulis* since the 1970́s. The Pacific oyster *Crassostrea gigas* is considered to be resistant to the disease and appears to have mechanisms to avoid infection. Most studies carried out on the invertebrate immune system focus on the role of hemolymph, although mucus, which covers the body surface of molluscs, could also act as a barrier against pathogens. In this study, the *in vitro* effect of mucus from the oyster species *Ostrea edulis* and *C. gigas* on *B. ostreae* was investigated using flow cytometry. Results showed an increase in esterase activities and mortality rate of parasites exposed to mucus from both oyster species. In order to better understand the potential role of mucus in the defense of the oyster against parasites such as *B. ostreae*, liquid chromatography and tandem mass spectrometry were used to describe and compare mucus protein composition from both species. In all oyster species, pallial mucus contains a high level of proteins; however, *O. edulis* mucus produced a variety of proteins that could be involved in the immune response against the parasite, including Cu/Zn extracellular superoxide dismutase, thioxiredoxin, peroxiredon VI, heat shock protein 90 as well as several hydrolases. Conversely, a different set of antioxidant proteins, hydrolases and stress related proteins were identified in mucus from *C. gigas*. Our results suggest an innate immunity adaptation of oysters to develop a specific response against their respective pathogens. The mucosal protein composition also provides new insights for further investigations into the immune response in oysters.

Graphical abstract



Highlights

► Mucus protein from oysters can interfere in the infection and viability of pathogens. ► Phagocytosis of *Bonamia ostreae* parasites decreases after mucus exposition. ► *O. edulis* mucus presents higher inhibitory effect than *C. gigas* mucus. ► Differential mucus composition of the species is related to pathogen recognition and inhibition. ► Adaptation of the responses against the specific pathogens of each species is suggested.

Keywords : *Bonamia ostreae, Crassostrea gigas, Ostrea edulis*, flow cytometry, immune response, proteome

1. Introduction

Bonamia ostreae is a protistan parasite belonging to the phylum Haplosporidia (Adl et al. 2012), which includes over 40 described species with representatives infecting a wide range of molluscan hosts (Arzul and Carnegie, 2015). *B. ostreae* was first described by Pichot et al. (1980) in France and, together with *Marteilia refringens,* has contributed to the decline of the flat oyster, *Ostrea edulis*, populations in Europe during the last three

decades (Vanbanning 1987; Figueras 1991; Montes et al. 1992; Arzul et al. 2011). Although the *B. ostreae* life cycle is not completely known, the parasite can infect different life stages of *O. edulis,* and oysters older than 2 years appear to be more susceptible to infection (Lynch et al. 2005; Lallias et al. 2008; Arzul et al. 2011). The parasite can also be transmitted directly from infected to uninfected oysters (Elston et al. 1987; Mialhe et al. 1988; Hervio et al. 1995). Because *O. edulis* maintain their larvae in the pallial cavity after liberation of female gametes previously fertilized by externally released sperm, it was suggested, after observation by *in situ* hybridization and detection of the parasite by PCR in those larvae, that *B. ostreae* can effectively be vertically transmitted (Arzul et al. 2011).

Experimental work suggested that *Crassostrea gigas* is a reservoir for *B. ostreae*. Using *in vitro* approaches, Lynch et al. (2010) demonstrated that *C. gigas* can contain parasite cells after exposure to infected *O. edulis* oysters and is a potential carrier of the disease. Infection with *B. ostreae* occurs during the entire year, but its dynamics present a seasonal pattern, including two annual prevalence peaks in winter/spring and autumn (Grizel, 1985; Montes, 1990; Vanbanning, 1990; Engelsma et al. 2010).

Although no clear correlation has been observed between the disease and environmental parameters like salinity and temperature, higher prevalence is observed in winter following low summer temperatures and high summer salinity (Engelsma et al. 2010; Flannery et al. 2014). Oysters maintained at low temperature (10ºC) had a higher prevalence of infection with *B. ostreae* and lower oyster hemocyte activity compared to oysters maintained at higher temperatures (20ºC). This suggests that low temperatures may affect defense capacities of the oyster and/or the capacity of the parasite to infect

new oysters (Cochennec, 2002; Arzul and Carnegie, 2015). In addition, free living *B. ostreae* cells showed significantly lower survival rate at 25ºC than at 4ºC and 15ºC (Arzul et al. 2009). Relatively cold temperatures could decrease the metabolic rate of free-living parasites, delaying mortality and augmenting the ability of the parasite to disperse throughout nearshore marine ecosystems (Ben-Horin et al. 2015).

B. ostreae survives and multiplies within hemocytes after phagocytosis (Chagot et al. 1992; Mourton et al. 1992; Montes et al. 1994) and, although mechanisms used by the parasite to enter and infect a new host are unknown, parasite cells seem to evade direct feeding and digestion of the host by initiating infections outside the digestive tract, most often in the pallial organs. Considering that these parasites initially infect the gill epithelium before invading nearby connective tissues (Montes et al. 1994; Ben-Horin et al. 2015), *B. ostreae* first must evade external barriers including mucus and epithelia. When these barriers are breached, the second internal line of defense involving cellular and soluble (humoral) hemolymph components come into play (Amstrong, 2006; Binias, 2014).

Mucus covers the body surface of molluscs, and is composed of water, mucins, electrolytes, epithelial and blood cells and a wide range of bioactive molecules produced by mucus-secreting cells (Schachter, 1982; Espinosa and Allam, 2016). Mucus protects epithelial cells not only against physicochemical injuries but also against infectious agents (Pales Espinosa et al. 2016). The role of mucus as a defense mechanism has been investigated in invertebrate species including the sea stars *Marthasterias glacialis* and *Asterias rubens* (Canicattí and D'Ancona, 1990; Stabili and Pagliara, 2009; Hennebert et al. 2015), snails (Iguchi et al. 1982), the polychaete *Laeonereis acuta* (Moraes et al.

2006), and the bivalves *Mytilus edulis*, *Crassostrea virginica* and *C. gigas* (Pales Espinosa et al. 2010; Jing et al. 2011; Pales Espinosa et al. 2014; Pales Espinosa et al. 2016).

The effect of oyster mucus was evaluated in infection of the oyster *C. virginica* by protozoan parasite *Perkinsus marinus* (Pales Espinosa et al. 2016). This parasite, responsible for massive mortality in *C. virginica* in eastern and southern North America, has no effect on the Pacific oyster *C. gigas*. Supplementation of *P. marinus* cultures with mucus from both species showed contrasting results; while mucus of *C. virginica* enhanced the growth and virulence of the parasite, mucus from *C. gigas* strongly inhibited the growth of *P. marinus* cultures. The characterization of the mucosal secretions from three different organs of *C. virginica* resulted in identification of more than 50 proteins involved in host-microbe interactions (Pales Espinosa et al. 2016).

The aim of the present study was to evaluate the role of mucus in the interactions between the oysters *O. edulis* and *C. gigas* and the intracellular parasite *B. ostreae* under different temperature regimes. *B. ostreae* was incubated in presence of mucus from *O. edulis* and *C. gigas* at four different temperatures and parasite viability was estimated by flow cytometry using propidium iodide (PI) as a mortality marker and fluorescein diacetate (FDA) to measure non-specific esterase activities which are related to cell metabolic rate (Gilham and Lehner, 2005). The capacity of mucus-exposed parasites to infect *O. edulis* hemocytes was evaluated by light microscopy after an *in vitro* contact experiment. Complementary liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to characterize and compare the proteomic composition of the mucus produced by both oyster species. We provide new insights into the role of mucus in host-

parasite interactions and, more specifically, into the susceptibility or resistance of oysters to the parasite *B. ostreae*.

2. Material and methods

2.1. *B. ostreae* exposure to mucus from *O. edulis* and *C. gigas*.

2.1.1. Oysters and mucus collection

Two-year-old flat oysters, *O. edulis,* were collected from Quiberon Bay (Brittany, France) and maintained in the quarantine facilities of the Ifremer Laboratory in La Tremblade, France until use (more than 30 days). Two-year-old *C. gigas* oysters were produced and maintained in these Ifremer facilities. Both species were maintained in raceways (120L) with a constant flow of seawater enriched in phytoplankton (*Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis succica*) at ambient seawater temperature. As the experiment was replicated several times, oysters were used during the months of July 2015 (sea water temperature average 20ºC), November 2015 (15ºC) and April 2016 (12ºC).

Mucus was obtained from both oyster species following the protocol described by Espinosa et al. (2009). Briefly, mucus was carefully collected from gills, mantle and labial palps using sterile cotton-tipped swabs. Swabs were then immersed in 10 mL ice-cold filtered sea water (FSW). Tubes containing swabs were placed at 4°C for 1 h on a rotating shaker. Swabs were removed and the fluid was then centrifuged (1000g, 30 min, 4°C), filter sterilized (0.22 µm syringe filters) and maintained at -80°C until use. A 25-µL aliquot of mucus solution was used to determine protein concentration with a Pierce BCA protein assay reagent kit (Bio-Rad) following manufacturer's recommendations.

For each experiment of parasite exposure to mucus, a pool of 10 individuals of each species was used to produce mucus, while 10 individual samples were collected from each species for LC-MS/MS analysis.

2.1.2. Parasite purification and species determination

B. ostreae was purified according to the protocol developed by Mialhe et al. (1988) with some modifications (Prado-Alvarez et al. 2013). Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After homogenization of all the organs except the adductor muscle, parasites were concentrated by differential centrifugation on sucrose gradients, collecting the interphase between the gradient of sucrose 20%-60%. Finally, the purified *B. ostreae* cells were suspended in FSW and counted using a Malassez-cell chamber.

Cells that were not used in the experiment were held at -20°C for parasite species confirmation by PCR. Real time PCR assays using the primer pairs BOSTRE-F/BOSTRE-R and BEXIT-F/ BEXIT-R (Ramilo et al. 2013) yielded positive results for *B. ostreae* and negative results for *B. exitiosa*.

2.1.4 *In vitro* exposure of *B. ostreae* to oyster mucus

Previous tests showed that 3-hour challenges allow observation of the effect of mucus on parasite viability and metabolism rate. Therefore, triplicate samples of 10^6 *B. ostreae* cells in 200 µL of 1-mg/ml protein mucus suspensions from *O. edulis* and *C. gigas* were incubated at 10, 15, 20 and 25°C for 3 hours. Control samples consisted of *B. ostreae* cells in FSW that were incubated in similar temperature conditions.

All exposure experiments were repeated 5 times. For each experiment, *B. ostreae* was isolated from a different infected oyster and the pool of mucus was also prepared from a different oyster batch (Tables S1, S2).

2.1.5. Flow cytometry analysis

Hemocyte mortality was evaluated by incubating 200 µl of cell suspension with 10 µl of propidium iodide (PI, 1.0 g l^{-1} Interchim) for 30 min in the dark. Non-specific esterase activities were evaluated by adding 1 µl of fluorescein diacetate (FDA, 400 µmol l^{-1}, Molecular Probes) to 200 µl of a hemocyte suspension for 30 minutes in the dark at room temperature, with subsequent transfer to ice for 5 min to stop the reaction (Morga et al. 2009).

For each sample, 5000 events were counted using an EPICS XL 4 (Beckman Coulter) based on size discrimination. Results were depicted as cell cytograms and reported in log scale fluorescence levels for each marker used. Fluorescence depended on the monitored parameters: non-specific esterase activities were measured using green fluorescence (FL1) while cell mortality was measured using red fluorescence (FL3) in logarithm phase according to protocols developed by Arzul et al. (2009). Positive samples of heat-inactivated parasites were used to evaluate the efficiency of the markers according to Morga et al. (2009). Analysis of the histograms was done with Flowing Software version 2.5 (Turku Centre for Technology, Finland).

Results were expressed as fold change variations between control and challenge parasites (ratio challenge/control). Data are presented as standard error of the mean ± SEM for all studies. Mean and SD were calculated for each triplicate. Significance was concluded at $p < 0.05$. The analyses were performed using the software SPSS version 12

(IBM Analytics) using ANOVA one factor analysis and post hoc proofs based on HSD Tukey test.

2.2. Contact experiments between mucus exposed parasites and oyster hemocytes

2.2.1 Hemolymph collection

Hemolymph was withdrawn from the adductor muscle sinus of 10-12 oysters using a 1-ml syringe equipped with a 23G needle. To eliminate debris, the hemolymph samples were filtered through 70-µm nylon mesh and held on ice to prevent cell aggregation. The volume of hemolymph collected from each oyster was approximately 1 ml. Samples were pooled, and hemocyte counts were performed using a Malassez-cell chamber. Hemocyte concentration was adjusted to 10^6 cells ml^{-1} using 0.22 µm filtered sea water (FSW).

2.2.2. *In vitro* contact between *B. ostreae* and *O. edulis* hemocytes

The contact test consisted of measuring the phagocytic capacity of *O. edulis* hemocytes incubated with parasites previously exposed to mucus of *O. edulis* (0.5 and 1 mg/mL) at 15°C for 3 h. Hemocytes were incubated with parasites at 15°C at a ratio of 10:1 parasites per hemocyte for 1 h. Control samples were hemocytes exposed to untreated parasites. The experiments were performed 3 times using three different pools of hemocytes and with three replicates for each test.

After 1 h of incubation, 100 µl of each cell suspension was cytocentrifuged (100 g, 4°C, 1 min), stained with Hemacolor (Merck) and examined using light microscopy. Percentage of phagocytosis was evaluated by examining 200 hemocytes on each slide.

2.3. Proteomic analysis of mucus by LC-MS/MS

2.3.1. Mucus preparation and LC-MS/MS analysis

Mucus from 10 *C. gigas* and 10 *O. edulis* was collected individually with cotton swabs and processed as described in 2.1.1. Protein concentration was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific Inc.). The volume containing 20 µg of protein was solubilized in 2x Laemmli buffer (Bio Rad). A quantity of 6 µg of each sample was applied on a 10% acrylamide SDS-PAGE gel for a short migration and cleaning purposes. After entering the resolving gel, separation was stopped. The gels were stained with colloidal blue overnight and de-stained in 25 mmol/L ammonium bicarbonate 50%, rinsed twice in ultrapure water and shrunk in acetonitrile (ACN) for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with trypsin solution (10 ng/µL in 40 mmol/L NH_4HCO_3 and 10% ACN), rehydrated at 4ºC for 10 min, and incubated overnight at 37 ºC. Gel pieces were then incubated for 15 min in 40 mmol/L NH_4HCO_3 and 10% ACN at room temperature on a rotary shaker. The supernatant was collected, and an extraction solution (H_2O/ACN/HCOOH (47,5:47,5:5)) was added onto gel slices for 15 min. The extraction step was repeated twice. Supernatants were collected in a volume of 60 µl and the peptide mixture was analyzed on a Q-Exactive LC-MS/MS (Thermo Fisher). Ten microliters of peptide digests were loaded onto a 300-µm-inner diameter × 5-mm C18 PepMapTM trap column (LC Packings) at a flow rate of 20 µL/min. The peptides were eluted from the trap column onto an analytical 75-mm id × 25-cm C18 Pep-Map column (LC Packings) with a 4–40% linear gradient of solvent B in 108 min (solvent A was 0.1% formic acid in 5% ACN, and solvent B was 0.1% formic acid in 80% ACN). The separation flow rate was set at 300 nL/min. The mass spectrometer operated in positive ion mode at a 1.8-kV needle voltage

and a 44-V capillary voltage. Data were acquired in a data-dependent mode alternating an FTMS scan survey over the range m/z 350–1600 and twelve ion trap MS/MS scans with higher-energy collision-induced dissociation (HCD) as activation mode. MS/MS spectra were acquired using a 2-m/z unit ion isolation window and normalized collision energy of 27. Mono-charged ions and unassigned charge-state ions were rejected from fragmentation. Dynamic exclusion duration was set to 30 s.

2.3.2. Database search and result processing

Data were searched by SEQUEST through Proteome Discoverer 2.3 (Thermo Fisher Scientific Inc.) against a NCBI protein database restricted to the Ostreidae family (140,823 entries – April 2019). Database included 75,152 entries from *C. gigas*, 60,643 from *C. virginica*, 996 from *C. sikamea*, 494 from *C. ariakensis*, 448 from *C. angulata*, 278 from *O. edulis* and 2,812 from other oyster species. The search parameters were established according to Fessart et al. (2014). Peptide validation was performed using Percolator algorithm (Kall et al. 2007) and only "high confidence" peptides were retained corresponding to a 1% False Positive Rate at peptide level.

Gene Ontology (GO) data of the 1498 differentially expressed proteins (DEPs) from *O. edulis* and *C. gigas* were obtained using the application PANNZER2 (Törönen et al. 2018). The GO accession numbers were then uploaded into the REVIGO application (Supek et al. 2011) to classify the proteins according to their molecular function using the semantic similarity measure from Resnik (normalized) against whole Uniprot database.

2.3.3 Label-free quantitative data analysis

Raw LC–MS/MS data were imported in Progenesis QI 2.0 (Non-Linear Dynamics). Data processing included the following steps: (i) Feature detection, (ii) Feature alignment across the 20 LC–MS/MS runs, (iii) Volume integration for 2–6 charge-state ions, (iv)

Normalization on total protein abundance (volume sum in each sample), (v) Import of sequence information, (vi) ANOVA test at peptide level and filtering for features p<0.05, (vii) Calculation of protein abundance (sum of the volume of corresponding peptides), and (viii) ANOVA test at protein level and filtering for features p<0.05. Proteins were grouped according to the parsimony principle to establish the minimal protein list covering all detected peptides. Only non-conflicting features and unique peptides were considered for calculation at the protein level. Quantitative data were considered for proteins quantified by a minimum of 2 peptides.

2.3.4 Protein expression statistical analysis

Data are presented as standard error of the mean ± SEM for all studies. Statistical differences between *C. gigas* and *O. edulis* data were analyzed using an ANOVA with GraphPad Prism 4 software. A *p* value of 0.05 or lower with a fold change greater than 2 or lower than 0.5 was considered significant. Differences in protein and peptide number were evaluated by the software SPSS version 12 (IBM Analytics) using Student t-test for independent samples. Significance was concluded at $p < 0.01$.

3. Results

3.1. *Effect of mucus on parasite viability*

Parasite mortality ranged from 6% to 39% in control conditions depending on the experiment and the temperature of incubation, although no significant difference was observed between tested temperatures (Table S1).

B. ostreae exposed for 3 hours to mucus from *O. edulis* and *C. gigas* showed a differential response compared to control samples in all tests (Fig.1). The parasite mortality rate was significantly higher when exposed to *O. edulis* mucus than when exposed to FSW

(control) and *C. gigas* mucus at all tested temperatures (Fig. 1A, Table S1). Although mortality also increased when the parasite was exposed to *C. gigas* mucus, the differences observed were not significant compared to control samples.

In the presence of mucus from both oyster species, the percentage of parasites displaying non-specific esterase activities (EA) increased (Fig. 1B, Fig. S2). This increase was significant at the four tested temperatures when *B. ostreae* was exposed to *O. edulis* mucus. However, only *B. ostreae* samples exposed to *C. gigas* mucus at 25ºC were significantly different against both control and *O. edulis* samples (Fig. 1B, Table S2).

Figures S2 and S3 show the comparison between conditions (FSW, *C. gigas* mucus and *O. edulis* mucus exposure) on density and histograms plots after PI and FDA labelling, respectively. A noticeable increase in the number of positive PI labelled cells was observed after *C. gigas* mucus exposure (Fig. S2B) compared to FSW (Fig. S2A) and *O. edulis* mucus (Fig. S2C). The circle population, shown in figure S2, is smaller after mucus exposure while a longer tail appeared. The conformation of *B. ostreae* cells changes after death due to post-mortem cellular changes; complexity increases and size decreases (Arzul et al. 2009). Regarding FDA-histogram, both mucus conditions (Fig. S3B and Fig. S3C) showed a higher number of events in the EA positive area compared to FSW (Fig. S3A).

3.2. *In vitro* exposure of *O. edulis* hemocytes and *B. ostreae* cells previously exposed to mucus

Following 3 hours exposure at 15ºC with *O. edulis* mucus at 0.5 and 1 mg/mL of protein concentration, parasites were incubated with hemocytes of *O. edulis* (ratio 10:1) for 1 hour. Light microscope observations showed a decrease in percentage of phagocytized parasites ($p<0.05$) when parasite cells were previously exposed to mucus (Fig. 2). In

control conditions, 13.67 ± 2.9% (mean ± S.E.M) of hemocytes phagocytized at least one parasite whereas 8.67 ± 0.76% and 7.83 ± 1.53% of hemocytes had internalized parasites after being exposed to 0.5 mg/mL and 1 mg/mL of mucus proteins, respectively.

3.3. Analysis of protein content of O. edulis and C. gigas mucus by LC-MS/MS

The analysis of the mucus protein content from 10 *C. gigas* and 10 *O. edulis* allowed identification of 2,400 unique proteins in *C. gigas* and 985 in *O. edulis*. The number of proteins identified per individual oyster was significantly lower on average (2.27 fold; $p<0.008$) in *O. edulis* than in *C. gigas* (Table S3).

3.3.1. Comparison of proteins identified in *O. edulis* and *C. gigas*

Results of the protein analysis were compared between *O. edulis* and *C. gigas* to identify common and specific proteins. Only proteins with at least two unique peptide hits were used for this comparison, reducing the number of total identified proteins to 1,498 (Supplementary datafile 1). Only 289 proteins that were differentially expressed at a statistically significant level between both species were considered in the study. Among those proteins, 24 were exclusive to *O. edulis,* another 51 proteins were expressed at a significantly higher level in *O. edulis* than in *C. gigas* (fold change > 2). Sixty-nine proteins were expressed at a significantly higher level in *C. gigas* (fold change < 0.5), and 145 proteins were found only in *C. gigas* (Supplementary datafile 1). Most of the proteins successfully identified were not differentially expressed between the two species (Fig. 3). Of these, 16.5% were common to *C. gigas* and *O. edulis* mucus and appeared to be related to hyproxanthine dehydrogenase activity; 15.52% were related to angiostatin binding; 8.24% to spermine synthase activity; and 6.99% to translation activator activity among others (Fig. 4A). Proteins only identified in *O. edulis* and those expressed at a significantly higher level in *O. edulis* (Fig. 4B) were mostly involved in protein

dimerization activity (17.96%), peptidase activity (14.36%) and oxidoreductase activity (12.6%). Finally, proteins only identified in *C. gigas* and significantly more expressed in *C. gigas* were mostly related to transcription factor binding (21.77%), sulfotransferase activity (12.16%) and kynureininase activity (11.66%) (Fig. 4C).

Proteins with an important role in immune recognition and immune responses were selected for discussion, among them 12 exclusive proteins of *Ostrea edulis* (Table 1), 25 differentially expressed proteins, 12 of them with high expression in *O. edulis* and 13 with high expression (Table 2) in *C. gigas* and 31 exclusive proteins from *C. gigas* were selected (Table 3).

Discussion

Effect of mucus on parasite viability

Mucus from invertebrates has a wide range of functions, among them acting as a barrier against injury and external agents such as bacteria and parasites (Fisher, 1992; Pales Espinosa et al. 2016). We estimated the effect of mucus on the flat oyster parasite *B. ostreae* by measuring mortality and non-specific esterase activities of parasites 3 hours after exposure to oyster mucus at different temperatures. Temperature is known to influence the dynamics of the infection with *B. ostreae* and its survival and might have an effect on parasite-mucus interactions (Arzul et al. 2009; Engelsma et al. 2010; Flannery et al. 2014; Arzul and Carnegie, 2015). At all tested incubation temperatures, *B. ostreae* survival decreased after exposure to mucus of *O. edulis* (susceptible host) and *C. gigas* (resistant host). The parasite mortality rate was always significantly higher when exposed to mucus from *O. edulis* compared to mucus from *C. gigas*. Moreover, parasites showed a significant increase of non-specific esterase activities after exposure to mucus

of each oyster species compared to control samples; however, the difference between *C. gigas* and *O. edulis* was only significant at 25ºC. The increase of non-esterase activities is not consistent with the increase of mortality. Taking into account that the dye FDA is also a viability marker (Peak et al. 2010) the increase of mortality should be followed by a decrease in esterase activity (Agusti et al. 1998). Measurement of esterase activities by FDA does have some limitations; a high quantity of extracellular esterases can give a high background and lead to false positives (Boyd et al. 2008). Also, some research has shown that, in certain conditions, cells can be stained by both dyes (PI and FDA) due to limitations of the technique (Boyd et al. 2008). *Schistosoma japonicum* cells exposed to aurafonin displayed cells stained either by PI or FDA while other compounds do not provoke such event (Peak et al. 2010). Additionally, several problems have been documented in the assessment of viability of human islets of Langerhans cells where intense extracellular staining and differential membrane integrity can affect the staining with FDA/PI dyes (Barnett et al. 2004; Boyd et al. 2008).

The increase of esterase activities could be explained by activation of parasite metabolism, including esterase activation by the mucus, a release of esterase proteins outside damaged cells or an effect of mucus on the cell membrane of the parasite leading to the entry of both dyes (FDA and PI) at the same time.

In tested conditions, our results suggest that *O. edulis* mucus is more efficient at inhibiting *B. ostreae* than *C. gigas* mucus, and that mucus effect from *C. gigas* does not explain its resistance to the parasite. Conversely, *P. marinus* displayed lower growth in presence of mucus of the resistant species *C. gigas*, compared to the susceptible *C. virginica.* Mucus of *C. virginica* enhanced parasite growth and virulence (Pales Espinosa et al. 2013; Pales Espinosa et al. 2014).

As previously described, *C. gigas* may act as a carrier of *B. ostreae*; parasites can be found in the extracellular fluid and tissues of oysters without known consequences, indicating that of *Bonamia* parasites could survive outside of the hemocytes and in non-typical hosts (Lynch et al. 2010). The involvement of *C. gigas* in the dispersion of parasite cells should be further evaluated. These observations are in accordance with the low mortality rate observed in *B. ostreae* cells exposed to *C. gigas* mucus. Mucus from *C. gigas* does not seem to have developed specific mechanisms to resist *B. ostreae* but, on the other hand, this species apparently has some mechanisms to avoid infection and propagation of the parasite. Additionally, in our study, the history of the tested oysters was different and possibly could explain the differential mortality rate of *B. ostreae* parasites when exposed to mucus from each species. *Ostrea edulis* over the age of 2 years were collected from Quiberon Bay, an area endemic for *B. ostreae.* Oysters were, therefore, not naïve, having been in contact with the parasite previously, and could have developed some tolerance. Also, the oysters from this location could be naturally selected by pressure of the disease, explaining the high activity of *O. edulis* mucus against *B. ostreae*. On the contrary, *C. gigas* oysters were produced and maintained at Ifremer facilities where no infection by *B. ostreae* has been recorded. Tested cupped oysters have thus never been in contact with the parasite suggesting that mechanisms of resistance are intrinsic to the species.

In our experimental conditions, temperature did not significantly modify the effect of mucus on parasite survival and metabolism (except at 25°C for mucus from *C. gigas*). These results are in accordance with results from previous work showing no effect of temperature on parasite survival during the first 12 hours of incubation whereas a

higher mortality rate was observed for *B. ostreae* incubated at 25ºC after 24 h (Arzul et al. 2009).

Effect of mucus on the internalization of *B. ostreae* in hemocytes

Although *O. edulis* mucus increases parasite mortality, no more than 52% of *B. ostreae* were killed after 3 h of exposure in the tested conditions, suggesting that most of parasites are able to pass through this barrier and interact with hemocytes. Previous work showed that *B. ostreae* can modify the phagocytosis capacity of hemocytes to facilitate its own internalization (Morga et al. 2008).

The present study demonstrates that incubating the parasite with mucus at two different protein concentrations (0,5 and 1 mg/mL) decreases its subsequent internalization in hemocytes. Mucus may impair the ability of parasites to infect the host (Stabili et al. 2015; Zanin et al. 2016) and, in the case of *B. ostreae*, a decrease in phagocytic rate could lead to an increase of resistance to the disease due to the inability of the parasite to spread through hemocytes (Morga et al. 2017; Gervais et al. 2018). It was previously shown that a treatment with cytochalasin B (known to block microfilament polymerisation) and heat inactivation of parasites decrease the engulfment of parasites by *O. edulis* hemocytes (Chagot et al. 1992; Gervais et al. 2016) indicating that *B. ostreae* participates actively in its own internalization. Our results suggest that *O. edulis* mucus inhibits the internalization of the parasite, at least during a short period of time. In a previous study, Gervais et al. (2016) observed that more live parasites were engulfed than inactivated ones by *O. edulis* hemocytes, while internalization rate in *C. gigas* hemocytes was similar, no matter the parasite status. Altogether, our results suggest an active mechanism of parasite internalization which

could be mitigated after a contact with *O. edulis* mucus, improving the defense of the oyster. The mechanisms involved in the internalization of the parasite are not completely understood but seem to involve several proteins including HSP90 and actin that, if blocked, reduce the percentage of infected hemocytes (Prado-Alvarez et al. 2013; Gervais et al. 2016).

Interspecific mucus protein comparison

Considering the differential effects of mucus from both oyster species observed on parasite survival, further effort was undertaken to compare their protein compositions and to identify potential candidates involved in the interactions between mucus and parasite. For that purpose, mucus was collected individually from 10 uninfected *O. edulis* and 10 *C. gigas,* which were held for at least 2 months in the same environmental conditions. Differences in protein mucus composition were expected, not only between both oyster species but also among individuals of the same species.

To our understanding this is the first proteome profile obtained from mucus of these two oyster species; the only previous study identified the mucus proteome of one species, the eastern oyster *C. virginica* (Pales Espinosa et al. 2016). The *C. virginica* study reported several proteins involved in immune recognition, immune activation and cell signaling, microbe neutralization or nonspecific response to stress.

We identified more than 2,400 different proteins in *C. gigas* samples and approximately 1353 in *O. edulis* samples. This important difference could be explained by the low number of *O. edulis* genome data in public databases. Moreover, only four reference proteomes from molluscs are available in Uniprot database: one gastropod (*Lottia gigantea*), two bivalves (*C. gigas* and *Patinopecten yessoensis*) and one cephalopod (*Octopus bimaculoides*), which limit the protein identification of *O. edulis* peptides.

As many as 1,320 proteins were identified in both species with at least two entries in our constructed database. Some of these proteins were exclusive to one species or showed significantly different expression levels between the species. Twenty-four proteins were exclusively found in *O. edulis* while 145 proteins were exclusive to *C. gigas*. A total of 69 proteins had a basal expression 2-fold higher in *C. gigas*, while a total of 51 proteins had a basal expression of 2-fold higher in *O. edulis*. Among these 120 proteins, several are related to immune function, immune recognition and response to stress.

Twenty-four proteins were exclusive to *O. edulis* and another 51 were differentially up-expressed in *O. edulis* with respect to *C. gigas*. Several were related to immune recognition, microbe neutralization or to detoxification and stress function. Among them, there were 3 hydrolases including lysozyme, alpha-L-fucosidase and puromycin-sensitive aminopeptidase. Lysozyme is an important part of the immune defense of invertebrates due to its muramidase activity against bacteria. Usually, lysozyme is highly expressed in mucus from labial palps and gills and also plasma, playing a role in digestion and protection against external agents (Xue et al. 2004; Itoh et al. 2007; Soudant et al. 2013). Alpha-L-phucosidase is an enzyme associated with carbohydrate metabolism, removing L-fucose residues in oligosaccharide chains (Artigaud et al. 2015), but it may also have a role in bacteria recognition and binding of fucosylated polysaccharides (Megson et al. 2015). The purimycin-sensitive aminopeptidase (PSA) is a peptidase involved in the degradation of proteins via proteasome pathway and autophagy. This peptidase, expressed at 28-fold higher levels in *O. edulis* than *in C. gigas,* is uniquely able to digest short polyglutamine peptides in humans (Menzies et al. 2010). In *C. gigas*,

Autophagy is activated during a viral and bacterial infection (Moreau et al. 2015; Picot et al. 2019).

Several proteins related to oxidation/reduction processes such as extracellular superoxide dismutase (EcSOD), peroxiredoxin, catalase and thioredoxin were also highly expressed in *O. edulis*. Two cathepsins (β and Z) were also up-regulated in *O. edulis*. Cathepsin β and Cu/Zn EcSOD were previously found to be over-expressed in hemocytes from *O. edulis* selected for their resistance to *B. ostreae* infection, suggesting their possible involvement in the defense and eventually resistance mechanisms against the parasite (Morga et al. 2012).

Additionally, three different thioredoxin (Trx) proteins were identified in *O. edulis* mucus, two of which were found to be exclusive to these *O. edulis* samples. Trx proteins are ubiquitous antioxidant enzymes that play important roles in health related cellular processes such as redox control, protein protection against aggregation or inactivation or programmed cell death, catalyzing the reduction of disulfide bonds (Collet and Messens, 2010). A completely different set of antioxidant proteins were found in *C. gigas*. Among them were cavortin, dominin, thioredoxin and both Mn and Cu/Zn extracellularsuperoxide dismutase (EcSOD). Dominin is the major plasma protein in *Crassostrea virginica*, representing up to 40% of eastern oyster plasma and extrapallial fluid (Itoh et al. 2011). This protein as well as cavortin, the major plasma protein of *C. gigas,* has a Cu/Zn SOD domain and is involved in several functions such as antioxidation, wound repair, metal transport and shell mineralization (Scotti et al. 2007; Itoh et al. 2011). The genus *Crassostrea* shares some common extracellular proteins with high antioxidant activity produced mainly by hemocytes that also have binding properties for bacteria, LPS and lipid A molecules (Gonzalez et al. 2005; Scotti et al. 2007).

Stress related proteins such as HSP90 and universal stress proteins were previously found to be differentially expressed in *O. edulis* after an infection with the parasite in different conditions and tissues (Martin-Gomez et al. 2008; Morga et al. 2012; Prado-Alvarez et al. 2013; Morga et al. 2017) completing the set of proteins involved in immune function.

Altogether, these results suggest high antioxidant and binding properties of mucus from both species. The higher expression of Cu/Zn EcSOD, PrxVI, thioredoxin as well as, HSP90 in *O. edulis* mucus could explain the greater effect against *B. ostreae* cells in comparison with *C. gigas* samples, which present a different set of antioxidant proteins. These proteins seem to have a major role in the immune response against *Bonamia* cells in *O. edulis*.

Some proteins differentially expressed at higher levels in *C. gigas* were directly involved in immune activation, cell signaling and microbe neutralization. Two different isoforms of ferritin were over-represented in *C. gigas* mucus. These proteins play a key role in the innate immune response of the organisms. During infections, pathogens use different mechanisms to acquire iron from their host for their growth and multiplication, while hosts attempt to withhold it from the pathogens to inhibit their growth and dissemination (Zheng et al. 2010; Maldonado-Aguayo et al. 2015). Studies carried out on shrimp have shown the involvement of ferritin in the protection against white spot syndrome virus (Huang et al. 2016). The over-expression of both proteins in *C. gigas* mucus could suggest an adaptive response of this oyster species to viral infections such as infection with OsHV-1, a virus that has been associated with high mortality of this oysters species since 1991 (Arzul et al. 2001).

Two lectins and one antimicrobial peptide were also over-expressed in *C. gigas*. Fucolectin and peptidoglycan recognition protein L (PRPL) are two proteins involved in external agent recognition and PRPL can have peptidase activity (Itoh and Takahashi, 2009).

Proteases and protease inhibitors are among the differentially expressed proteins found between the two oyster species. The protease Cathepsin L is a cysteine protease that is up-regulated after bacterial challenge in the oyster *Pinctada fucata* (Ma et al. 2010), and the cysteine rich antimicrobial peptide "cystein rich protein 1" is one of the major antimicrobial agents of oyster defense (Liu et al. 2008). Protease inhibitors Cystatin-β and metalloproteinase inhibitor 3 are part of the immune defense mechanism of *C. gigas* mucus. Cystatin-β is a potent inhibitor of cysteine proteinases in invertebrates (Premachandra et al. 2012) while metalloproteinase inhibits metalloproteinase zinc dependent endopeptidase. This protein is mainly secreted by hemocytes and could be an important factor in immune defense of oysters (Montagnani et al. 2001).

Conclusion

In this study, we exposed the protozoan parasite *B. ostreae* to mucus from the susceptible oyster species *O. edulis* and the resistant species *C. gigas*. In tested conditions, mucus from both species induces an increase of esterase activity and mortality in parasite cells. Additionally, exposure to mucus decreases the level of infection of hemocytes *in vitro,* probably by inhibiting the ability of the parasite to activate its own internalization. These findings led us to explore and compare mucus protein composition from both oyster species. Several proteins were differentially expressed including proteins involved in immune functions, cell signaling, microbe

neutralization or autophagy. Mucus can act as an effector against pathogens and can actively participate in the immune response of invertebrates. The difference in mucus protein composition between the two oyster species could partly explain their differential susceptibility to diseases and notably to infection with *B. ostreae*. While the main pathogenic disease problems in *O. edulis* are caused by the protozoan parasites *B. ostreae*, *B. exitiosa* and *Marteilia refringens*, *C. gigas* is primarily affected by an oyster herpervirus (OsHV-1) and, more recently, by the bacterium *Vibrio aestuarianus*. Both oyster species may have developed immune mechanisms adapted to avoid their primary pathogens. The notable difference in antioxidant protein composition between the species may explain their differential susceptibility to *Bonamia* sp. infection. Although these differences alone do not explain why one species is susceptible and the other is resistant to *B. ostreae* infection, this differential protein expression will be valuable information for further research into the innate immune system of the two species.

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Fig. 1. *Bonamia ostreae* exposure to oyster mucus. (A) Fold change of mortality rate (IP-stained cells) in parasites exposed to oyster mucus for 3h compared to control samples (parasites in FSW). (B) Fold change of non-specific esterase activities (positive cells) in *B. ostreae* cells exposed to oyster mucus for 3 h compared to control samples. Axis Y represent the fold change while Axis X the temperature. Data are presented as mean ± SEM. One-way ANOVA followed by Tuckey test showed (**a**) $p< 0.05$ differences founded compared to control data at the same temperature. (**b**) $p< 0.05$ Differences in *C. gigas* species in comparison with *O. edulis* at the same temperature. (*****) $p< 0.05$ differences among temperatures taking 10ºC as control, only significate differences were found in *C. gigas* EA at 25ºC.

Fig. 2 Percentage of hemocytes which have phagocyted at least one *B. ostreae* after 1 hour incubation. Control samples were parasite cells incubated at 15ºC for 3h in FSW and then exposed to *O. edulis* hemocytes (ratio 10:1) for 1h. Experimental samples were *B. ostreae* cells exposed to a differential concentration of *O. edulis* mucus protein for 3h at 15ºC, and then exposed to *O. edulis* hemocytes (ratio 10:1) for 1h. * $p<0.05$ compared to control data.

Fig. 3. Number of exclusive and common proteins between *O. edulis* and *C. gigas* mucus according their differential expression pattern fold change.

Fig. 4. GO classification by their molecular function of the total proteins of both species (A); differential expressed proteins from *O. edulis* (B); differential expressed proteins from *C. gigas* (C) according REVIGO software.

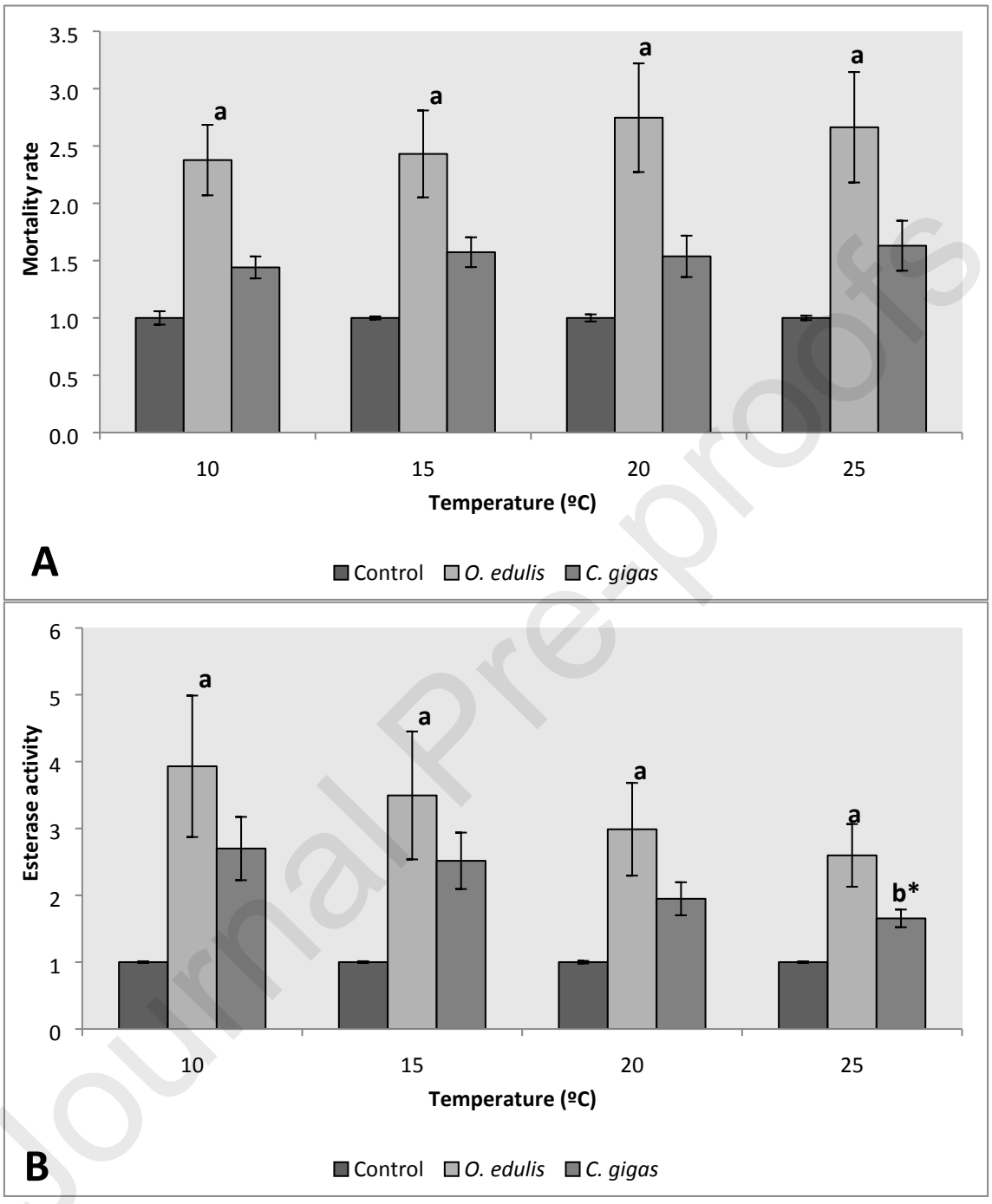


Fig. 1

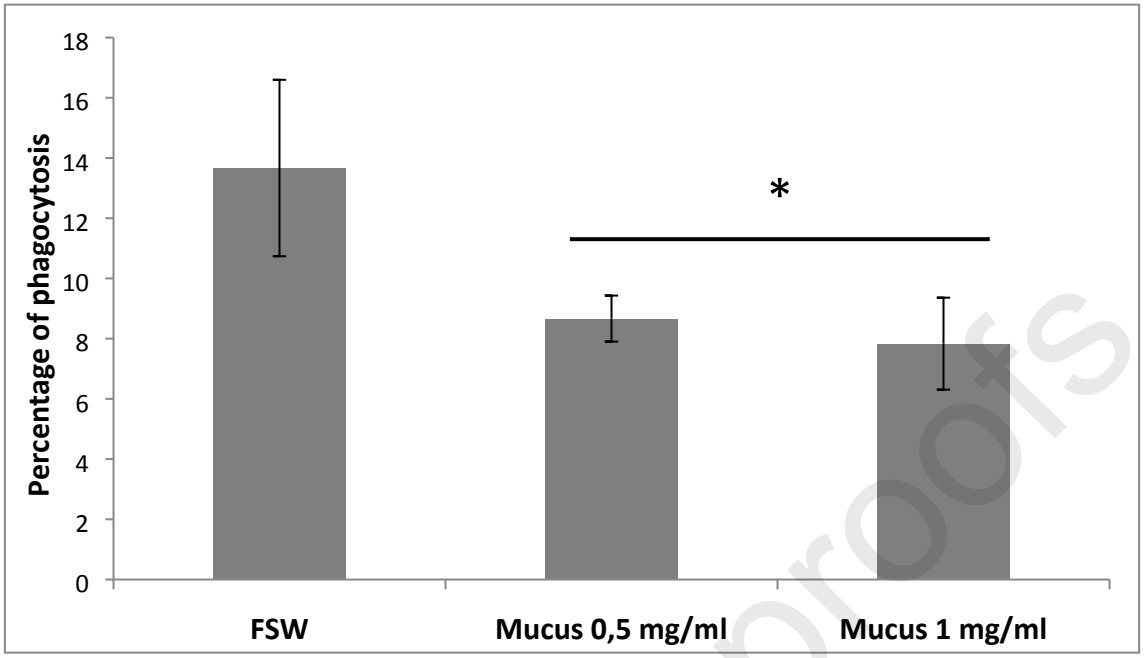


Fig. 2

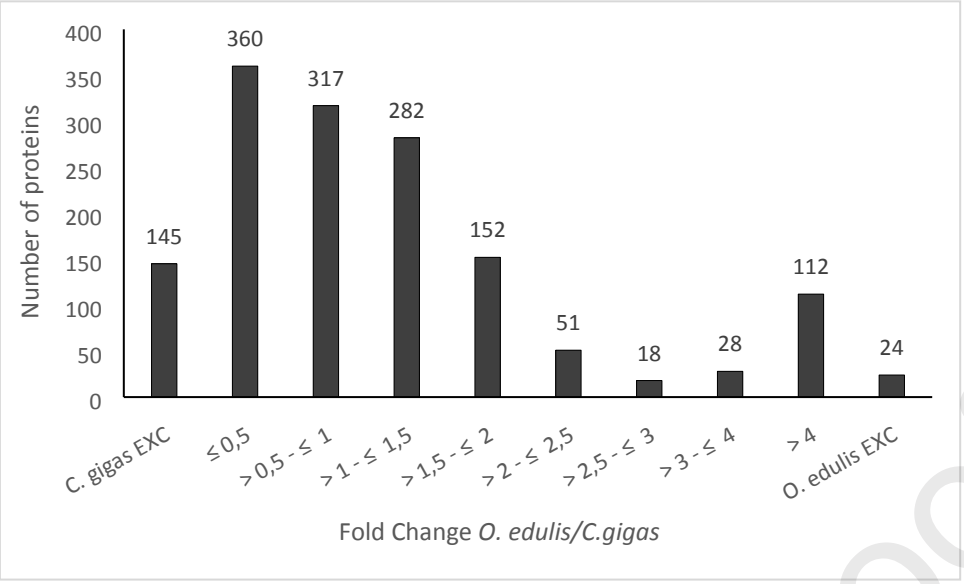
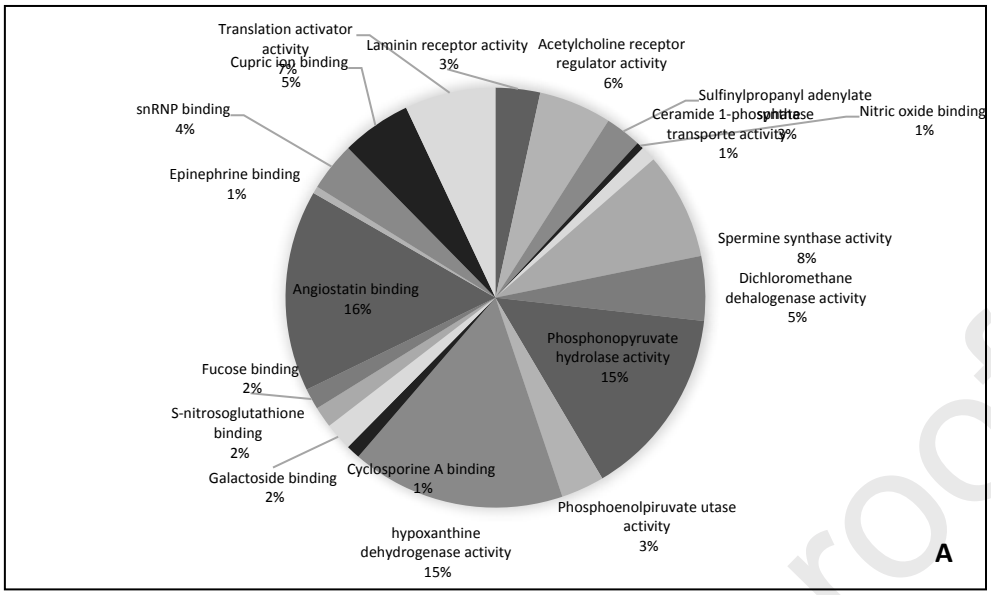
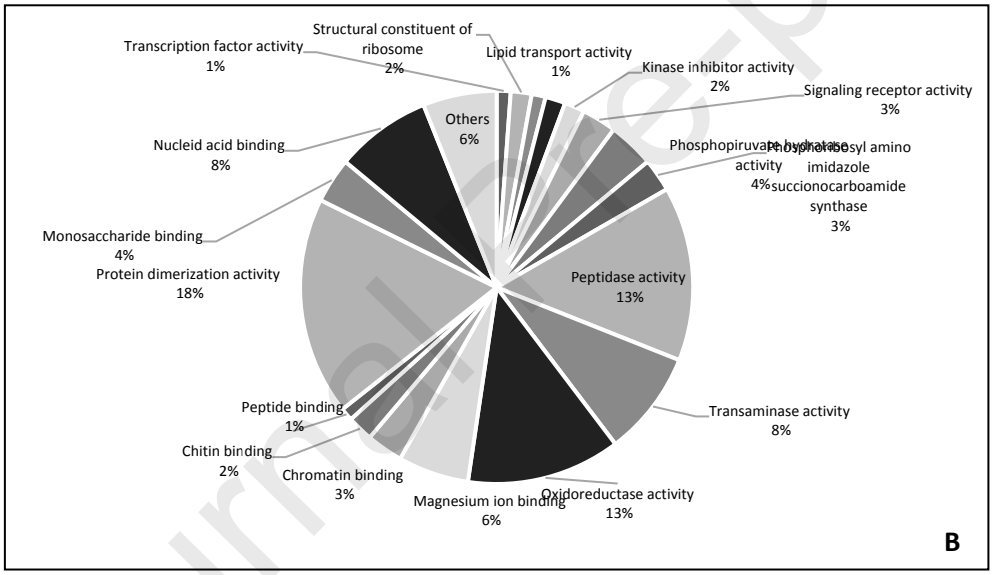


Fig. 3





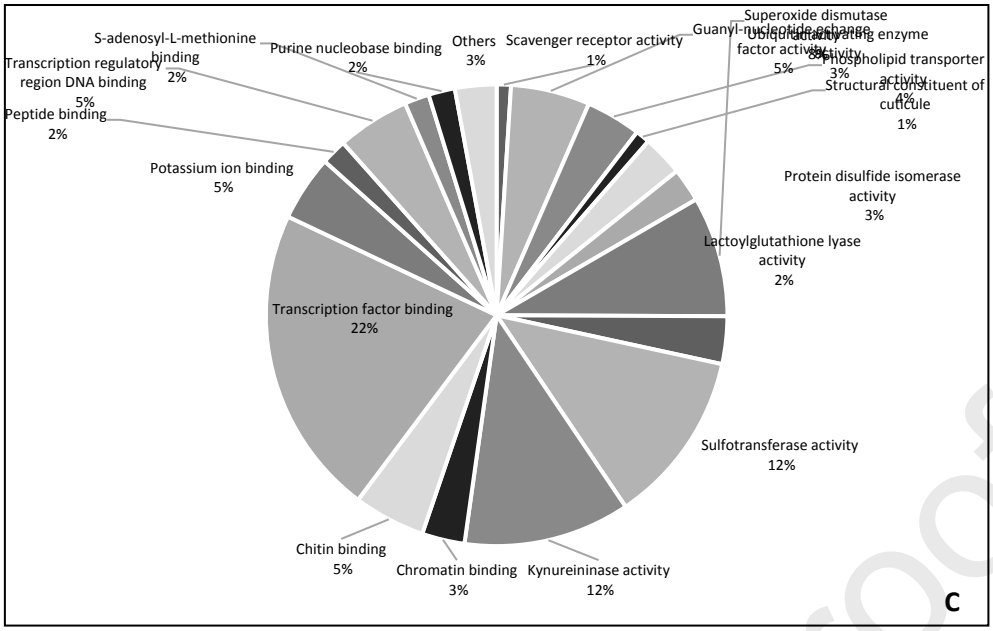


Fig. 4

Table 1. List of the 12 exclusive proteins selected for their immune function from *O. edulis*. Gene Ontology terms: P-biological process; F-molecular function; C-cellular component.

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Biological process GO-id, description		Molecular function GO-id, description		Cellular component GO-id, description	
gi|295136549|gb|ADF80415.1| extracellular Cu-Zn superoxide dismutase [Ostrea edulis]	1000	3,80811E-17	GO:0006801	superoxide metabolic process	GO:0046872	metal ion binding		
gi|1242834524|ref|XP_022298339.1| universal stress protein PHOS32-like [Crassostrea virginica]	1000	3,80811E-17	GO:0071918	urea transmembrane transport	GO:0015204	urea transmembrane transporter activity	GO:0005737	cytoplasm
			GO:0042558	pteridine-containing compound metabolic process	GO:0016787	hydrolase activity	GO:0016021	integral component of membrane
gi|47496785|dbj|BAD19060.1| lysozyme [Ostrea edulis]	1000	3,80811E-17	GO:0042742	defense response to bacterium	GO:0003796	lysozyme activity	GO:0005576	extracellular region
			GO:0019835	cytolysis	GO:0004568	chitinase activity	GO:0005634	nucleus
			GO:0051672	catabolism by organism of cell wall peptidoglycan in other organism	GO:0047968	glyoxylate dehydrogenase (acylating) activity		
gi|229485193|gb|ACQ73550.1| peroxiredoxin 6 [Saccostrea glomerata]	1000	3,80811E-17	GO:0098869	cellular oxidant detoxification	GO:0051920	peroxiredoxin activity	GO:0005623	cell
			GO:0045454	cell redox homeostasis	GO:0004601	peroxidase activity		
			GO:0055114	oxidation-reduction process	GO:0005515	protein binding		
gi|375073556|gb|AFA34340.1| anexin 11a, partial [Ostrea edulis]	1000	3,80811E-17	GO:0050819	negative regulation of coagulation	GO:0005544	calcium-dependent phospholipid binding	GO:0012506	vesicle membrane
			GO:0030855	epithelial cell differentiation	GO:0005509	calcium ion binding	GO:0048471	perinuclear region of cytoplasm
			GO:2000483	negative regulation of interleukin-8 secretion	GO:0048306	calcium-dependent protein binding	GO:0031965	nuclear membrane
gi|1242796885|ref|XP_022338846.1| LOW QUALITY PROTEIN: catalase-like [Crassostrea virginica]	1000	3,80811E-17	GO:0042744	hydrogen peroxide catabolic process	GO:0004096	catalase activity	GO:0005777	peroxisome
			GO:0006979	response to oxidative stress	GO:0020037	heme binding	GO:0016021	integral component of membrane
			GO:0098869	cellular oxidant detoxification	GO:0046872	metal ion binding		
gi|1242785489|ref|XP_022332735.1| bifunctional glutamate/proline--tRNA ligase-like [Crassostrea virginica]	1000	3,80811E-17	GO:0006433	prolyl-tRNA aminoacylation	GO:0004827	proline-tRNA ligase activity	GO:0017101	aminoacyl-tRNA synthetase multienzyme complex
			GO:0006424	glutamyl-tRNA aminoacylation	GO:0004818	glutamate-tRNA ligase activity	GO:0005737	cytoplasm
			GO:0044539	long-chain fatty acid import into cell	GO:0005524	ATP binding	GO:0097452	GAIT complex
gi|1242810058|ref|XP_022345866.1| uncharacterized protein	1000	3,80811E-17	GO:0098869	cellular oxidant detoxification	GO:0004601	peroxidase activity	GO:0016021	integral component of membrane
			GO:0055114	oxidation-reduction process	GO:0020037	heme binding		

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Biological process GO-id, description		Molecular function GO-id, description		Cellular component GO-id, description	
LOC111138280 [Crassostrea virginica]								
gi|1242784183|ref|XP_022332033.1| heat shock protein HSP 90-beta-like [Crassostrea virginica]	1000	3,80811E-17	GO:0006457	protein folding	GO:0051082	unfolded protein binding	GO:0034751	aryl hydrocarbon receptor complex
			GO:1901389	negative regulation of transforming growth factor beta activation	GO:0005524	ATP binding	GO:1990565	HSP90-CDC37 chaperone complex
			GO:1901799	negative regulation of proteasomal protein catabolic process	GO:0019900	kinase binding	GO:0044294	dendritic growth cone
gi|1242782317|ref|XP_022331028.1| methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial [Crassostrea virginica]	1000	3,80811E-17	GO:0055114	oxidation-reduction process	GO:0004491	methylmalonate-semialdehyde dehydrogenase (acylating) activity		
gi|1242837345|ref|XP_022299798.1| thioredoxin reductase 1, cytoplasmic-like isoform X1 [Crassostrea virginica]	1000	3,80811E-17	GO:0098869	cellular oxidant detoxification	GO:0004791	thioredoxin-disulfide reductase activity	GO:0005654	nucleoplasm
			GO:0045454	cell redox homeostasis	GO:0050660	flavin adenine dinucleotide binding	GO:0005829	cytosol
			GO:0022900	electron transport chain	GO:0015035	protein disulfide oxidoreductase activity	GO:0005739	mitochondrion
gi|1242772135|ref|XP_022325563.1| thioredoxin domain-containing protein 3 homolog isoform X1 [Crassostrea virginica]	1000	3,80811E-17	GO:0006165	nucleoside diphosphate phosphorylation	GO:0004550	nucleoside diphosphate kinase activity	GO:0005623	cell
			GO:0006228	UTP biosynthetic process	GO:0030246	carbohydrate binding		
			GO:0006183	GTP biosynthetic process				

Table 2. List of the selected 25 differentially expressed proteins between *O. edulis* and *C. gigas* according their immune function. Fold change >2 and <0.5. (ANOVA-F test, FDR <0.05). Gene Ontology terms: P-biological process; F-molecular function; C-cellular component.

Query header	Abundance Ratio: (OE/CG)	P Value (OE/CG)	Biological process GO-id, description		Molecular function GO-id, description		Cellular component GO-id, description	
gi|375073721|gb|AFA34417.1|	190.186	0,000123	GO:0006436	tryptophanyl-tRNA aminoacylation	GO:0004830	tryptophan-tRNA ligase activity	GO:0005829	cytosol
tryptophanyl tRNA synthetase WARS, partial [Ostrea edulis]			GO:0010835	regulation of protein ADP-ribosylation	GO:0005524	ATP binding	GO:0005634	nucleus
			GO:0045765	regulation of angiogenesis	GO:0019210	kinase inhibitor activity	GO:0032991	protein-containing complex
gi|1242834325|ref|XP_022298238.1|	171.377	0,000178	GO:0051016	barbed-end actin filament capping	GO:0003779	actin binding	GO:0008290	F-actin capping protein complex
F-actin-capping protein subunit alpha-1-like isoform X1 [Crassostrea virginica]			GO:0030036	actin cytoskeleton organization	GO:0044877	protein-containing complex binding	GO:0030863	cortical cytoskeleton
			GO:0051490	negative regulation of filopodium assembly	GO:0046982	protein heterodimerization activity	GO:0061645	endocytic patch
gi|1242784483|ref|XP_022332194.1|	136.444	0,000387	GO:0006108	malate metabolic process	GO:0030060	L-malate dehydrogenase activity	GO:0005829	cytosol
malate dehydrogenase, cytoplasmic-like [Crassostrea virginica]			GO:0006099	tricarboxylic acid cycle	GO:0051287	NAD binding	GO:0043209	myelin sheath
			GO:0005975	carbohydrate metabolic process	GO:0047860	diiodophenylpyruvate reductase activity	GO:0070062	extracellular exosome
gi|1242772356|ref|XP_022325682.1|	84.492	0,001767	GO:0006436	tryptophanyl-tRNA aminoacylation	GO:0004830	tryptophan-tRNA ligase activity	GO:0005829	cytosol
tryptophan--tRNA ligase, cytoplasmic-like [Crassostrea virginica]			GO:0010835	regulation of protein ADP-ribosylation	GO:0005524	ATP binding	GO:0005634	nucleus
			GO:0045765	regulation of angiogenesis	GO:0019210	kinase inhibitor activity	GO:0032991	protein-containing complex
gi|1242768297|ref|XP_022323515.1|	39.304	0,014218	GO:0010951	negative regulation of endopeptidase activity	GO:0004866	endopeptidase inhibitor activity	GO:0005615	extracellular space
CD109 antigen-like isoform X1 [Crassostrea virginica]			GO:0050829	defense response to Gram-negative bacterium	GO:0008233	peptidase activity	GO:0016021	integral component of membrane
			GO:0045087	innate immune response	GO:0008061	chitin binding		
gi|1242788961|ref|XP_022334575.1|	38.734	0,004881	GO:0005975	carbohydrate metabolic process	GO:0004563	beta-N-acetylhexosaminidase activity	GO:0005764	lysosome
beta-hexosaminidase subunit beta-like isoform X1 [Crassostrea virginica]			GO:0001525	angiogenesis	GO:0102148	N-acetyl-beta-D-galactosaminidase activity	GO:0001669	acrosomal vesicle
			GO:0006689	ganglioside catabolic process	GO:0008478	pyridoxal kinase activity	GO:0005615	extracellular space
gi|295136547|gb|ADF80414.1|	36.769	0,016768	GO:0019430	removal of superoxide radicals	GO:0004784	superoxide dismutase activity	GO:0005829	cytosol
Cu-Zn superoxide dismutase [Ostrea edulis]			GO:0055114	oxidation-reduction process	GO:0046872	metal ion binding	GO:0005634	nucleus
			GO:0051597	response to methylmercury	GO:0004601	peroxidase activity	GO:0005739	mitochondrion
gi|1242796833|ref|XP_022338818.1|	28.577	0,030109	GO:0006508	proteolysis	GO:0004177	aminopeptidase activity	GO:0005829	cytosol

Query header	Abundance Ratio: (OE/CG)	P Value (OE/CG)	Biological process GO-id, description		Molecular function GO-id, description		Cellular component GO-id, description	
puromycin-sensitive aminopeptidase-like [Crassostrea virginica]			GO:0071456	cellular response to hypoxia	GO:0008237	metallopeptidase activity	GO:0070062	extracellular exosome
			GO:0043171	peptide catabolic process	GO:0008270	zinc ion binding	GO:0005634	nucleus
gi|388571210|gb|AFK73701.1|	28.268	0,00924	GO:0032880	regulation of protein localization	GO:0005080	protein kinase C binding	GO:1990630	IRE1-RACK1-PP2A complex
receptor of activated kinase C [Ostrea edulis]			GO:0050765	negative regulation of phagocytosis	GO:0030292	protein tyrosine kinase inhibitor activity	GO:0001891	phagocytic cup
			GO:0033137	negative regulation of peptidyl-serine phosphorylation	GO:0008656	cysteine-type endopeptidase activator activity involved in apoptotic process	GO:0030496	midbody
gi|1242786338|ref|XP_022333200.1|	25.221	0,010055	GO:0055114	oxidation-reduction process	GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0005759	mitochondrial matrix
aldehyde dehydrogenase, mitochondrial-like [Crassostrea virginica]			GO:0006068	ethanol catabolic process	GO:0070404	NADH binding		
			GO:0006470	protein dephosphorylation	GO:0008138	protein tyrosine/serine/threonine phosphatase activity		
gi|1242832504|ref|XP_022297304.1|	18.057	0,005213	GO:0006004	fucose metabolic process	GO:0004560	alpha-L-fucosidase activity	GO:0005764	lysosome
alpha-L-fucosidase-like [Crassostrea virginica]			GO:0016139	glycoside catabolic process	GO:0003950	NAD+ ADP-ribosyltransferase activity	GO:0005615	extracellular space
			GO:2000535	regulation of entry of bacterium into host cell	GO:0003676	nucleic acid binding	GO:0016021	integral component of membrane
gi|405967869|gb|EKC32989.1|	12.906	0,017174	GO:0050715	positive regulation of cytokine secretion	GO:0030246	carbohydrate binding	GO:0016021	integral component of membrane
Aggrecan core protein [Crassostrea gigas]			GO:0002221	pattern recognition receptor signaling pathway	GO:0038187	pattern recognition receptor activity		
			GO:0042742	defense response to bacterium	GO:0005509	calcium ion binding		
gi|405974461|gb|EKC39104.1|	0.108	0,027711	GO:0016567	protein ubiquitination	GO:0004842	ubiquitin-protein transferase activity	GO:0016021	integral component of membrane
E3 ubiquitin-protein ligase MIB2 [Crassostrea gigas]			GO:0006471	protein ADP-ribosylation	GO:0003950	NAD+ ADP-ribosyltransferase activity	GO:0005813	centrosome
			GO:0006468	protein phosphorylation	GO:0008270	zinc ion binding	GO:0005737	cytoplasm
gi|405963300|gb|EKC28886.1|	0.086	0,013174	GO:0043666	regulation of phosphoprotein phosphatase activity	GO:0019888	protein phosphatase regulator activity	GO:0000159	protein phosphatase type 2A complex
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform [Crassostrea gigas]			GO:0007165	signal transduction	GO:0019211	phosphatase activator activity	GO:0005829	cytosol
			GO:0035307	positive regulation of protein dephosphorylation	GO:0004721	phosphoprotein phosphatase activity	GO:0005634	nucleus
gi|762074407|ref|XP_011438883.1|	0.062	0,009118	GO:0006879	cellular iron ion homeostasis	GO:0004322	ferroxidase activity	GO:0005623	cell
PREDICTED: ferritin, lower subunit [Crassostrea gigas]			GO:0006826	iron ion transport	GO:0008199	ferric iron binding		
			GO:0055114	oxidation-reduction process				
gi|1139843823|ref|XP_019918948.1|	0.06	0,0101	GO:0006511	ubiquitin-dependent protein catabolic process	GO:0070003	threonine-type peptidase activity	GO:0019773	proteasome core complex, alpha-subunit complex

Query header	Abundance Ratio: (OE/CG)	P Value (OE/CG)	Biological process GO-id, description		Molecular function GO-id, description		Cellular component GO-id, description	
PREDICTED: proteasome subunit alpha type-6 [Crassostrea gigas]			GO:0051092	positive regulation of NF-kappaB transcription factor activity	GO:0004175	endopeptidase activity	GO:0005634	nucleus
			GO:0010499	proteasomal ubiquitin-independent protein catabolic process	GO:0051059	NF-kappaB binding	GO:0005737	cytoplasm
gi|405959611|gb|EKC25626.1|	0.06	0,019052	GO:0010951	negative regulation of endopeptidase activity	GO:0004869	cysteine-type endopeptidase inhibitor activity	GO:0005829	cytosol
Cystatin-B [Crassostrea gigas]			GO:0030216	keratinocyte differentiation	GO:0002020	protease binding	GO:0005615	extracellular space
			GO:0018149	peptide cross-linking	GO:0030674	protein binding, bridging	GO:0001533	cornified envelope
gi|405962293|gb|EKC27985.1|	0.05	0,027413	GO:0019430	removal of superoxide radicals	GO:0004784	superoxide dismutase activity	GO:0005739	mitochondrion
Superoxide dismutase [Mn], mitochondrial [Crassostrea gigas]			GO:0055114	oxidation-reduction process	GO:0046872	metal ion binding	GO:0070013	intracellular organelle lumen
			GO:0051597	response to methylmercury	GO:0042803	protein homodimerization activity	GO:0044446	intracellular organelle part
gi|1139752565|ref|XP_011426105.2|	0.042	0,017775	GO:0055114	oxidation-reduction process	GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0005576	extracellular region
PREDICTED: uncharacterized protein LOC105327371 [Crassostrea gigas]			GO:0005975	carbohydrate metabolic process	GO:0030246	carbohydrate binding		
					GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds		
gi|762107912|ref|XP_011436573.1|	0.029	7,27E-05	GO:0030887	positive regulation of myeloid dendritic cell activation	GO:0030246	carbohydrate binding	GO:0005886	plasma membrane
PREDICTED: perlucin-like protein [Crassostrea gigas]			GO:0038094	Fc-gamma receptor signaling pathway	GO:0034987	immunoglobulin receptor binding	GO:0016021	integral component of membrane
			GO:0045087	innate immune response				
gi|762108130|ref|XP_011436688.1|	0.02	5,7E-05	GO:0071493	cellular response to UV-B	GO:0046872	metal ion binding	GO:0005737	cytoplasm
PREDICTED: cysteine-rich protein 1 [Crassostrea gigas]			GO:0010043	response to zinc ion	GO:0008301	DNA binding, bending	GO:0071944	cell periphery
			GO:0008630	intrinsic apoptotic signaling pathway in response to DNA damage	GO:0003680	AT DNA binding	GO:0016021	integral component of membrane
gi|1345541793|gb|AVG18814.1|	0.014	0,00095	GO:0005975	carbohydrate metabolic process	GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0005576	extracellular region
DM9-containing protein-2CgDM9CP-2 [Crassostrea gigas]			GO:0055114	oxidation-reduction process	GO:0030246	carbohydrate binding		
					GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds		
gi|762088186|ref|XP_011426403.1| PREDICTED: metalloproteinase inhibitor 3 [Crassostrea gigas]	0.005	1,73E-05	GO:0010951	negative regulation of endopeptidase activity	GO:0008191	metalloendopeptidase inhibitor activity		
gi|30039400|gb|AAP12558.1| cavortin, partial [Crassostrea gigas]	0.004	7,19E-06	GO:0006801	superoxide metabolic process	GO:0046872	metal ion binding		

Query header	Abundance Ratio: (OE/CG)	P Value (OE/CG)	Biological process GO-id, description	Molecular function GO-id, description	Cellular component GO-id, description
gi|1024262283|gb|ANB49597.1| dominin (Crassostrea brasiliana)	0.003	5,26E-06	GO:0006801 superoxide metabolic process	GO:0046872 metal ion binding	

Table 3. List of the 31 exclusive proteins selected for their immune function from *C. gigas*. Gene Ontology terms: P-biological process; F-molecular function; C-cellular component.

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Cellular component GO-id, description		Cellular component GO-id, description		Cellular component GO-id, description	
gi|1139840052|ref|XP_011456641.2| PREDICTED: zonadhesin-like [Crassostrea gigas]	0,001	3,81E-17	GO:0042730	fibrinolysis	GO:0004252	serine-type endopeptidase activity	GO:0005576	extracellular region
			GO:0048771	tissue remodeling	GO:1904854	proteasome core complex binding	GO:0031232	extrinsic component of external side of plasma membrane
			GO:0007596	blood coagulation	GO:1990405	protein antigen binding	GO:0098685	Schaffer collateral - CA1 synapse
gi|762078237|ref|XP_011452919.1| PREDICTED: bifunctional purine biosynthesis protein PURH [Crassostrea gigas]	0,001	3,81E-17	GO:0006164	purine nucleotide biosynthetic process	GO:0004643	phosphoribosylaminoimidazolecarboxamide formyltransferase activity	GO:0005829	cytosol
			GO:0098761	cellular response to interleukin-7	GO:0003937	IMP cyclohydrolase activity	GO:0005886	plasma membrane
			GO:0042060	wound healing	GO:0042803	protein homodimerization activity	GO:0070062	extracellular exosome
gi|762080824|ref|XP_011419107.1| PREDICTED: galactokinase-like [Crassostrea gigas]	0,001	3,81E-17	GO:0006012	galactose metabolic process	GO:0004335	galactokinase activity	GO:0005737	cytoplasm
			GO:0046835	carbohydrate phosphorylation	GO:0005534	galactose binding	GO:0070062	extracellular exosome
			GO:0061623	glycolytic process from galactose	GO:0005524	ATP binding	GO:0016020	membrane
gi|762103601|ref|XP_011434325.1| PREDICTED: uncharacterized protein LOC105333177 [Crassostrea gigas]	0,001	3,81E-17	GO:0010951	negative regulation of endopeptidase activity	GO:0004869	cysteine-type endopeptidase inhibitor activity	GO:0005764	lysosome
			GO:0006508	proteolysis	GO:0008234	cysteine-type peptidase activity	GO:0005615	extracellular space
			GO:0044257	cellular protein catabolic process	GO:0004175	endopeptidase activity	GO:0008180	COP9 signalosome
gi|405971257|gb|EKC36103.1| Uncharacterized protein y4xO [Crassostrea gigas]	0,001	3,81E-17	GO:0055114	oxidation-reduction process	GO:0050662	coenzyme binding		
					GO:0016491	oxidoreductase activity		
gi|405949999|gb|EKC18008.1| Glyoxalase domain-containing protein 4 [Crassostrea gigas]	0,001	3,81E-17	GO:0006481	C-terminal protein methylation	GO:0045296	cadherin binding	GO:0005739	mitochondrion
					GO:0004671	protein C-terminal S-isoprenylcysteine carboxyl O-methyltransferase activity	GO:0070062	extracellular exosome
gi|762073957|ref|XP_011436495.1| PREDICTED: copper transport protein ATX1-like [Crassostrea gigas]	0,001	3,81E-17	GO:0030001	metal ion transport	GO:0046872	metal ion binding	GO:0005829	cytosol
			GO:0006979	response to oxidative stress	GO:0016530	metallochaperone activity	GO:0005743	mitochondrial inner membrane
			GO:0034759	regulation of iron ion transmembrane transport	GO:0032767	copper-dependent protein binding	GO:0005634	nucleus
gi|762122063|ref|XP_011443977.1| PREDICTED: uncharacterized protein LOC105339896 [Crassostrea gigas]	0,001	3,81E-17	GO:0097194	execution phase of apoptosis	GO:0004197	cysteine-type endopeptidase activity	GO:0005737	cytoplasm
			GO:0006508	proteolysis				
			GO:0042981	regulation of apoptotic process				
gi|762097373|ref|XP_011431255.1|	0,001	3,81E-17	GO:0032510	endosome to lysosome transport via multivesicular body sorting pathway	GO:0008745	N-acetylmuramoyl-L-alanine amidase activity	GO:0031901	early endosome membrane
PREDICTED: UBX domain-containing protein 6 isoform X1 [Crassostrea gigas]			GO:0036503	ERAD pathway	GO:0008270	zinc ion binding	GO:0031902	late endosome membrane
			GO:0016236	macroautophagy	GO:0005515	protein binding	GO:0005765	lysosomal membrane
gi|1242740236|ref|XP_022330217.1| signal transducer and activator of transcription 5B-like isoform X1 [Crassostrea virginica]	0,001	3,81E-17	GO:0007165	signal transduction	GO:0003700	DNA-binding transcription factor activity	GO:0005634	nucleus

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Cellular component GO-id, description		Cellular component GO-id, description		Cellular component GO-id, description	
			GO:0071378	cellular response to growth hormone stimulus	GO:0003677	DNA binding	GO:0005737	cytoplasm
			GO:0006355	regulation of transcription, DNA-templated	GO:0019903	protein phosphatase binding	GO:0000785	chromatin
gi|405969114|gb|EKC34120.1| Thioredoxin domain-containing protein 3-like protein [Crassostrea gigas]	0,001	3,81E-17	GO:0006165	nucleoside diphosphate phosphorylation	GO:0004550	nucleoside diphosphate kinase activity	GO:0005623	cell
			GO:0006228	UTP biosynthetic process	GO:0004563	beta-N-acetylhexosaminidase activity		
			GO:0006183	GTP biosynthetic process				
gi|405952729|gb|EKC20506.1| Titin [Crassostrea gigas]	0,001	3,81E-17	GO:0009617	response to bacterium	GO:0005509	calcium ion binding	GO:0005604	basement membrane
			GO:0090497	mesenchymal cell migration	GO:0005201	extracellular matrix structural constituent	GO:0005938	cell cortex
			GO:0035122	embryonic medial fin morphogenesis	GO:0050839	cell adhesion molecule binding	GO:0030054	cell junction
gi|405976150|gb|EKC40668.1| Purine nucleoside phosphorylase [Crassostrea gigas]	0,001	3,81E-17	GO:0009116	nucleoside metabolic process	GO:0004731	purine-nucleoside phosphorylase activity	GO:0005829	cytosol
			GO:0034356	NAD biosynthesis via nicotinamide riboside salvage pathway	GO:0002060	purine nucleobase binding	GO:0005576	extracellular region
			GO:0034418	urate biosynthetic process	GO:0042301	phosphate ion binding	GO:0005634	nucleus
gi|405951422|gb|EKC19336.1| Eukaryotic translation initiation factor 3 subunit G-A [Crassostrea gigas]	0,001	3,81E-17	GO:0001732	formation of cytoplasmic translation initiation complex	GO:0003743	translation initiation factor activity	GO:0005852	eukaryotic translation initiation factor 3 complex
			GO:0075525	viral translational termination-reinitiation	GO:0005515	protein binding	GO:0016282	eukaryotic 43S preinitiation complex
			GO:0006508	proteolysis	GO:0004252	serine-type endopeptidase activity	GO:0033290	eukaryotic 48S preinitiation complex
gi|1139752128|ref|XP_019922308.1| PREDICTED: collagen alpha-1(XII) chain-like isoform X2 [Crassostrea gigas]	0,001	3,81E-17	GO:0007229	integrin-mediated signaling pathway	GO:0005201	extracellular matrix structural constituent	GO:0005581	collagen trimer
			GO:0010951	negative regulation of endopeptidase activity	GO:0004867	serine-type endopeptidase inhibitor activity	GO:0062023	collagen-containing extracellular matrix
			GO:0003429	growth plate cartilage chondrocyte morphogenesis			GO:0008305	integrin complex
gi|762139509|ref|XP_011453106.1| PREDICTED: 26S proteasome non-ATPase regulatory subunit 8 isoform X1 [Crassostrea gigas]	0,001	3,81E-17	GO:0009405	pathogenesis	GO:0090729	toxin activity	GO:0005838	proteasome regulatory particle
			GO:0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	GO:0005515	protein binding	GO:0005829	cytosol
			GO:0070498	interleukin-1-mediated signaling pathway			GO:0005654	nucleoplasm
gi|762126852|ref|XP_011446491.1| PREDICTED: cathepsin L1 [Crassostrea gigas]	0,001	3,81E-17	GO:0006508	proteolysis	GO:0008234	cysteine-type peptidase activity	GO:0005764	lysosome
			GO:0044257	cellular protein catabolic process	GO:0004175	endopeptidase activity	GO:0005615	extracellular space
			GO:0010951	negative regulation of endopeptidase activity	GO:0004869	cysteine-type endopeptidase inhibitor activity	GO:0016021	integral component of membrane
gi|762144051|ref|XP_011455290.1| PREDICTED: signal recognition particle subunit SRP68 [Crassostrea gigas]	0,001	3,81E-17	GO:0006614	SRP-dependent cotranslational protein targeting to membrane	GO:0030942	endoplasmic reticulum signal peptide binding	GO:0005786	signal recognition particle, endoplasmic reticulum targeting
			GO:0042493	response to drug	GO:0005047	signal recognition particle binding	GO:0005925	focal adhesion
gi|238768474|dbj|BAH66800.1| peptidoglycan recognition protein L [Crassostrea gigas]	0,001	3,81E-17	GO:0009253	peptidoglycan catabolic process	GO:0061783	peptidoglycan muralytic activity	GO:0005576	extracellular region
			GO:0016998	cell wall macromolecule catabolic process	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	GO:0016020	membrane
			GO:0045087	innate response	GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not		

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Cellular component GO-id, description		Cellular component GO-id, description		Cellular component GO-id, description	
						peptide) bonds, in linear amides		
gi|762094928|ref|XP_011429960.1|PREDICTED: fucolectin-like [Crassostrea gigas]	0,001	3,81E-17	GO:0051607	defense response to virus			GO:0005739	mitochondrion
gi|762073807|ref|XP_011435693.1| PREDICTED: interferon-induced protein 44 isoform X1 [Crassostrea gigas]	0,001	3,81E-17	GO:0009617	response to bacterium	GO:0005525	GTP binding	GO:0005737	cytoplasm
			GO:0006955	immune response				
			GO:0009615	response to virus				
gi|762132867|ref|XP_011449627.1| PREDICTED: caspase-3 [Crassostrea gigas]	0,001	3,81E-17	GO:0006508	proteolysis	GO:0004197	cysteine-type endopeptidase activity	GO:0005737	cytoplasm
			GO:0006915	apoptotic process	GO:0008373	sialyltransferase activity	GO:0012505	endomembrane system
			GO:0048696	regulation of collateral sprouting in absence of injury	GO:0016874	ligase activity	GO:0043231	intracellular membrane-bounded organelle
gi|1139856155|ref|XP_011423396.2| PREDICTED: uncharacterized protein LOC105325506 [Crassostrea gigas]	0,001	3,81E-17	GO:0032502	developmental process	GO:0048029	monosaccharide binding	GO:0005615	extracellular space
			GO:0043001	Golgi to plasma membrane protein transport	GO:0005507	copper ion binding	GO:0005581	collagen trimer
			GO:0007030	Golgi organization			GO:0016021	integral component of membrane
gi|405962631|gb|EKC28289.1| Soma ferritin [Crassostrea gigas]	0,001	3,81E-17	GO:0006879	cellular iron ion homeostasis	GO:0004322	ferroxidase activity	GO:0005623	cell
			GO:0006826	iron ion transport	GO:0008199	ferric iron binding	GO:0044422	organelle part
			GO:0055114	oxidation-reduction process	GO:0003700	DNA-binding transcription factor activity	GO:0043227	membrane-bounded organelle
gi|762113890|ref|XP_011439711.1| PREDICTED: proteasome subunit alpha type-1 [Crassostrea gigas]	0,001	3,81E-17	GO:0006511	ubiquitin-dependent protein catabolic process	GO:0070003	threonine-type peptidase activity	GO:0019773	proteasome core complex, alpha-subunit complex
			GO:0002862	negative regulation of inflammatory response to antigenic stimulus	GO:0004175	endopeptidase activity	GO:0005634	nucleus
			GO:0010499	proteasomal ubiquitin-independent protein catabolic process	GO:0001530	lipopolysaccharide binding	GO:0005813	centrosome
gi|1242742763|ref|XP_022343526.1| proteasomal ubiquitin receptor ADRM1-B-like [Crassostrea virginica]	0,001	3,81E-17	GO:0010950	positive regulation of endopeptidase activity	GO:0061133	endopeptidase activator activity	GO:0005634	nucleus
			GO:0043248	proteasome assembly	GO:0070628	proteasome binding	GO:0005737	cytoplasm
			GO:0006368	transcription elongation from RNA polymerase II promoter	GO:0043130	ubiquitin binding	GO:0000502	proteasome complex
gi|762146295|ref|XP_011456448.1| PREDICTED: 10 kDa heat shock protein, mitochondrial-like [Crassostrea gigas]	0,001	3,81E-17	GO:0006457	protein folding	GO:0005524	ATP binding	GO:0005737	cytoplasm
					GO:0051087	chaperone binding	GO:0070013	intracellular organelle lumen
					GO:0051082	unfolded protein binding	GO:0043231	intracellular membrane-bounded organelle
					GO:0046872	metal ion binding	GO:0044446	intracellular organelle part
gi|1139842567|ref|XP_019918643.1| PREDICTED: hsp90 co-chaperone Cdc37 [Crassostrea gigas]	0,001	3,81E-17	GO:0060330	regulation of response to interferon-gamma	GO:0019901	protein kinase binding	GO:1990565	HSP90-CDC37 chaperone complex
			GO:0098779	positive regulation of mitophagy in response to mitochondrial depolarization	GO:0097110	scaffold protein binding	GO:0005829	cytosol
			GO:0060338	regulation of type I interferon-mediated signaling pathway	GO:0031072	heat shock protein binding	GO:0070062	extracellular exosome
gi|405962104|gb|EKC27808.1| Pancreatic triacylglycerol lipase [Crassostrea gigas]	0,001	3,81E-17	GO:0006629	lipid metabolic process	GO:0052689	carboxylic ester hydrolase activity	GO:0005576	extracellular region
			GO:0006968	cellular defense response	GO:0016298	lipase activity	GO:1990777	lipoprotein particle

Query header	Abundance Ratio: (OE/CG)	P-Value: (OE/CG)	Cellular component GO-id, description	Cellular component GO-id, description	Cellular component GO-id, description
gi|405950524|gb|EKC18507.1| Heat shock 70 kDa protein 12A [Crassostrea gigas]	0,001	3,81E-17		GO:0005524 ATP binding	GO:0016021 integral component of membrane

Highlights

1. Mucus protein from oysters can interfere in the infection and viability of pathogens.
2. Phagocytosis of *Bonamia ostreae* parasites decreases after mucus exposition.
3. *O. edulis* mucus presents higher inhibitory effect than *C. gigas* mucus.
4. Differential mucus composition of the species is related to pathogen recognition and inhibition.
5. Adaptation of the responses against the specific pathogens of each species is suggested.

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