Morphological characterization and functional immune response of the carpet shell clam (*Ruditapes decussatus*) haemocytes after bacterial stimulation.

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Submitted to: Fish and Shellfish Immunology.

Date: October 2011

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Abstract

The morphology and functionality of *Ruditapes decussatus* haemocytes have been characterized by light microscopy and flow cytometry, leading to the identification of three different cellular subpopulations. Granulocytes were the largest cells, the hyalinocytes were smaller and contained fewer granules and the intermediate cells showed a size similar to hyalinocytes and a higher number of granules. The phagocytosis of different particles and the associated production of oxygen radicals were measured by flow cytometric methods. Granulocytes were the most active cells, followed by the intermediate cells and hyalinocytes. The effect of stimulation of haemocytes with lipopolysaccharide (LPS), with a heat inactivated bacterial mixture or with the infection of *Vibrio splendidus* on the cell viability and the expression of selected immune related genes were studied. While significant low levels of damaged cells were registered in LPS-stimulated cells, the treatment with dead bacteria or *V. splendidus* reduced cell viability 1 h, 3 h and 6 h after treatment. The stimulation of haemocytes with LPS and dead bacteria induced changes in the expression of defender against cell death (DAD-1), thrombin, prosaposin, inhibitor of apoptosis (IAP), factor B and C3 complement component.

Key words: *Ruditapes decussatus*, haemocyte, immune system, flow cytometry, phagocytosis, reactive oxygen radicals, cell viability, bacterial infection, gene expression.

1. Introduction

Haemocytes are circulating cells present in bivalve molluscs haemolymph. They are involved in both physiological processes and immune functions such as phagocytosis [1-3]. Since haemocytes have been broadly studied to determine the immune and physiological status of economically important bivalves [4-7] their classification and functionality might be of interest to better understand bivalve defence. The division of the haemocytes is based on the nature of the cytoplasmatic organelles, the acidification and abundance of cytoplasmatic granules and the morphology of the nucleus [8]. The haemocytes of several bivalve species were identified following Cheng´s criteria who classified them into granulocytes with numerous and large granules, and hyalinocytes with less or no granules in the cytoplasm [8-18].

In addition to morphological and cytochemical criteria, some cell separation techniques such as density gradients have been used to classify the haemocytic cells in *Crassostrea gigas*, *Crassostrea virginica*, *Mytilus edulis*, *Ostrea edulis* and *Argopecten irradians* [19-23]. The identification of subpopulations using monoclonal antibodies has been successful in *M. edulis* and *O. edulis* [24, 25] but not in *R. decussatus* [14]. Both cell types, granulocytes and hyalinocytes, are able to internalise foreign particles and pathogens by phagocytosis [15, 26-30]. However, it has been hypothesized that the presence of granules with hydrolytic enzymes could act to degrade the phagocytised particles suggesting that granulocytes have higher phagocytic activity than hyalinocytes [31].. Internalised material is also degraded by the action of the reactive oxidative species (ROS) released during the phagocytosis process. After primary and secondary reactions, toxic free radicals such as the superoxide anion (O^{2-}), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) are released [32, 33].

Phagocytosis and respiratory burst play an important role in bivalve defence [15, 27, 34] and are studied to determine the immune status against pathogens [4-7, 35, 36]. Although several methodologies have been applied to determine these cellular immune parameters, the application of the flow cytometry allows the simultaneous morphological and functional characterization of the cell populations. Different cell subpopulations of *O. edulis, Ruditapes philippinarum, C. virginica, M. edulis* and *Mytilus galloprovincialis* were characterised by flow cytometry as well as several immune parameters such as phagocytosis and oxidative burst [37-44]. The production of ROS has not been detected in the clams *Mya arenaria, Mercenaria mercenaria, R. decussatus* and *Scrobicularia plana* by the classical assays: luminol-dependent chemiluminiscence, the reduction of nitroblue tetrazolium (NBT) and the reduction of cytochrome-c [45-48]. However, the application of flow cytometry has recently allowed the detection of ROS production in the clam *M. mercenaria* [42].

Gram-negative bacteria are widely distributed in marine ecosystem, and they can affect the production of farmed bivalves, even when they are non pathogenic in many situations [49-54] Among them, *V. splendidus* and other related strains have been associated with larvae mortality both *in R. decussatus* and *C. gigas* [55-57]. The bivalve immune response against bacterial infection has been studied by experimental infections with pathogenic bacteria and stimulations with extracellular products and different components of the bacterial cell wall such as lipopolysacharide of gram-negative bacteria [36, 58, 59]. Several functional studies were carried out in bacterial infected bivalves, however the information regarding the molecular basis involved in the immune function, although increasing, is still scarce [4, 60, 61]. Recently, several genes have been identified by Suppression-Subtractive Hybridization to be involved in the immune functions of *R. decussatus* after parasitic infection and dead bacterial infection.

Among them, the defender against cell death (DAD-1), thrombin, prosaposin, inhibitor of apoptosis (IAP), factor B and C3 complement component were selected due to their involvement on death signaling and complement system activation [62-64].

The aim of the present work is the morphological and functional characterization of the different populations of haemocytes in *R. decussatus* by flow cytometry. Changes in the cell viability and in the selected gene expression profiles were determined after experimental infections with *V. splendidus* and stimulations with dead bacteria and LPS.

2. Materials and methods

2.1. Animals

Sexually immature adult carpet shell clam, *R. decussatus* (4-5 cm long) were obtained from a commercial shellfish farm and maintained in opened circuit with filtered sea water (FSW) at 15ºC for one week before the experiments. Animals were daily feeding with *Tetraselmis suecica* (10^7 cells/ml) and *Isochrysis galbana* (10^7 cells/ml). All animal experiments were conducted according the CSIC National Committee on Bioethics.

2.2. Morphological characterization of *R. decussatus* haemocytes

The characterization of the different cell populations in haemolymph from carpet shell clams was done by light microscopy and flow cytometry.

2.2.1. Light microscopy studies

Haemolymph was withdrawn without anti-aggregating solution through the adductor muscle with sterile needle and syringes after shell perforation. Each sample

was treated individually and maintained on ice until use. Haemolymph was diluted in filtered sea water (FSW) (1:5) and centrifuged at 55xg for 5 min in a Shandon Cytospin 4 cytocentrifuge (Thermo Scientific). Haemocytes were fixed with ethanol, stained with the Hemacolor kit (Merck) and mounted with DEPEX resin (BDH, Chemicals). Haemocytes were visualised using an Eclipse 80i light microscopy (Nikon) with Nomarski DIC prism to enhance the contrast in fresh non-stained samples. Photographs were taken with a digital camera DXM 1200 (Nikon).

2.2.2 Flow cytometry analysis

R. decussatus haemocytes subpopulations were identified by flow cytometry (FACSCalibur, BD) in density plots of relative size (forward-light-scatter, FSC-H) and complexity (side-light-scatter, SSC-H) in logarithmic scale. The different cell regions were located in density plot graphics by the Cell Quest Pro software (BD) using 1.0% of threshold and two smoothing passes. Haemolymph samples extracted from 12 clams were diluted in FSW (1:1) and individually analysed. A total of $2x10^6$ cells of each subpopulation were collected in a 50 ml Falcon tube (BD) using the cell sorter module and centrifuged at 82xg for 5 min. The cell pellet was resuspended in 1 ml of FSW and treated for light microscopy as described above.

2.3. Functional characterization of *R. decussatus* haemocytes

The functional characterization of the haemolymph cell populations was done by analyzing the ability to phagocyte different particles and the associated production of oxygen radicals.

147 **2.3.1. Phagocytosis assay**

148 Crude haemolymph samples (200 µl) were withdrawn as described in section
149 2.2.1 and maintained on ice for a maximum of 15 minutes before being dispensed into
150 96-wells plates. Four replicates of each haemolymph sample were made to compare the
151 phagocytosis of three different fluorescein-labelled (FITC) particles: latex microspheres
152 at $2.7x10^8$ particles/ml (1.3 µm, Molecular Probes), *Escherichia coli* at $2.7x10^{10}$
153 particles/ml (Sigma) and zymosan at $1.7x10^8$ particles/ml (Sigma). Labelled particles
154 were added at a ratio of 10:1 (particles: haemocyte). Control haemocytes were
155 maintained in FSW. After two hours of incubation at 15ºC samples were washed twice
156 with phosphate buffered saline (PBS). Attached cells were collected in PBS and stained
157 with Trypan blue (0.8% in PBS) to quench the adhered but non-phagocyted particles
158 fluorescence. Phagocytosis was assayed by flow cytometry after the measurement of
159 10000 events per sample. The experiment was repeated four times with 4 clams each
160 experiment. Results are shown as the mean ± standard deviation of the percentage of
161 cells that internalised at least one fluorescent particle. Data were analyzed using
162 Student's t-test and differences were statistically significant at $p<0.05$. Haemocytes
163 exposed to FITC-labelled particles were observed with an Eclipse 80i light microscopy
164 (Nikon) with Nomarski DIC prism and compared with haemocytes exposed to FSW.
165 Photographs were taken with a digital camera DXM 1200 (Nikon).
166

167 **2.3.2. Respiratory burst assay**

168 The production of oxygen radicals was measured by flow cytometry using the
169 2´,7´-dichlorofluorescein-diacetate probe (DCFH-DA , Molecular Probes). The DCFH-
170 DA diffuses into the cytoplasm where is blocked and hydrolysed to 2´,7´-
171 dichlorofluorescein (DCFH). After oxidation by the released oxygen radicals, the

hydrolysed form emits fluorescence that is detected in the FL1-H channel. Crude haemolymph samples (200 µl) were maintained on ice for 15 min maximum before being dispensed into 96-wells plates in triplicate. After 30 min of incubation at 15ºC in the dark for cell adhesion, the media was replaced with the DCFH-DA solution (1:1000 in FSW, 0.4% dimethyl sulfoxide) and incubated 10 min on ice. Haemocytes were rinsed twice before stimulation with zymosan (Sigma) at 0.5 mg/ml in FSW. ROS-inhibited samples were treated with superoxide dismutase (SOD, Sigma) at 300 U/ml just before the stimulus and FSW was used as control. Cells were incubated 30 min at 15ºC in the dark, and measured by flow cytometry after resuspending in PBS. The experiment was repeated five times with a total of 22 clams and 10000 events were measured per sample. Mean fluorescence index were calculated as the ratio of stimulated samples to the control. Results are shown as the mean ± standard deviation of the calculated mean fluorescence index in each region obtained in the four trials. Data were analyzed using Student's t-test and differences were statistically significant at $p<0.05$.

2.4. *In vitro* stimulation of *R. decussatus* haemocytes

LPS (Sigma, Aldrich) at a final concentration of 50 µg/ml in FSW, live *V. splendidus* and a mixture of heat inactivated bacteria (*Micrococcus luteus*, *V. splendidus* and *Vibrio anguillarum*) were used to stimulate clam haemocytes. *V. splendidus* were cultured overnight at room temperature (20ºC) in TSA supplemented with 1%NaCl. *V. splendidus* suspension was then prepared in FSW to obtain 10^6 CFU/ml (OD_{620} = 0.033). To prepare the mixture of inactivated bacteria, the strains were grown in appropriate medium (TSB for *M. luteus* and TSB supplemented with 1%NaCl for *Vibrio* spp.) and

196 equal amounts of culture medium with an OD_{620} = 0.035 were heat inactivated at 95ºC
197 for 10 min. Aliquots were maintained at -80ºC until use.
198 The immune response of the carpet shell clam haemocytes was characterized
199 measuring the effect of the stimulation on the different cell populations viability and
200 changes in the gene expression profile.
201
202 **2.4.1. Effect of the stimulants on the cell viability**
203 The viability of the different populations of haemocytes was analyzed after the
204 exposure to different stimuli using the fluorescent dye propidium iodide (PI) (BD,
205 Pharmingen), which penetrates through broken cell membranes. Haemolymph from 16
206 adult clams was extracted, pooled in 4 different samples and dispensed into 96-wells
207 plates (200 µl/well). Haemocyte were incubated 30 min at 15ºC. After adhesion,
208 haemocytes were stimulated with 100 µl of different solutions containing LPS (50
209 µg/ml), live *V. splendidus* (10^6 CFU/ml) or a mixture of diluted heat inactivated bacteria
210 diluted 1/3 in FSW before the experiments. Control haemocytes were incubated in FSW
211 in the same way than stimulated haemocytes. Cell cultures were incubated at 15 ºC and
212 1h, 3h and 6h after stimulation samples were stained with PI (25 µg/ml), incubated for
213 10 min in the dark and measured by flow cytometry. The experiment was repeated five
214 times. Fold-change units were calculated dividing the values obtained in stimulated
215 samples by the values obtained in the control. Results are shown as the mean ± SD of
216 the fold-change units obtained in the five trials. Data were analyzed using Student's t-
217 test and differences were statistically significant at $p<0.05$.
218
219 **2.4.2. Effect of the stimulants on the gene expression profile**

Haemolymph from twelve clams was extracted and pooled in 4 samples (3 clams each). Cells were dispensed on 24-well plates (1 ml/well) and incubated for 30 min at 15ºC. After adhesion, haemocytes were stimulated as previously described for 1 h, 3 h and 6 h. Haemocytes from each pool, treatment and sampling point were then collected and total RNA was extracted in 6 ml of Trizol reagent (Invitrogen) following the manufacturer's protocol. The purity and integrity of the RNA was evaluated in a ND-1000 Spectrophotometer (Nanodrop Technologies, USA). First-strand cDNAs were synthesized with SuperScript II (Invitrogen) using 1 µg of total RNA, treated with Turbo DNA-free (Ambion) to remove contaminating DNA. Specific primers were designed with Primer3 software (v. 0.4.0) and checked to ensure similar efficiencies in the amplification reaction (Table 1). Real time PCR was carried out in a 7300 Real Time PCR System (Applied Biosystems). A total of 0.5 µl of each primer (10 µM) was mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl.

Amplification was carried out at standard cycling conditions (95ºC for 10 min, followed by 40 cycles of 95ºC 15 s and 60ºC for 1 min). All reactions were carried out as technical triplicates. The comparative Ct method ($2^{-\Delta\Delta Ct}$ method) was used to determine the expression level of analyzed genes [65]. The expression of the candidate genes was normalized using the *R. decussatus* actin gene as a control housekeeping gene, which was constitutively expressed and not affected by the treatments. Fold units were calculated by dividing the normalized expression values obtained in stimulated samples by the normalized expression values obtained in the control at each sampling point. Data were analyzed using the Student's t-test. Results were expressed as the mean ± standard deviation of the four different samples and differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Morphological characterization of *R. decussatus* haemocytes

The visualization of fresh haemolymph samples by light microscopy allowed the description of three morphologically distinct subpopulations also supported by the presence of three different cell populations in FSC-H/SSC-H dot plots observed by flow cytometry. Due to the high variability between individuals, the cell population perimeters were slightly adjusted for each clam. The R1 included the biggest cells with high granularity. The R2 enclosed the smallest cells with low numbers of cytoplasmatic granules and the R3 was composed by cells with intermediate values of size and granularity (Figure 1A).

The morphological differences detected in each subpopulation by flow cytometry were verified after cell sorting, cytocentrifugation and fresh and stained observations under the light microscope. Each sorted region were photographed and compared with fresh haemocyte preparations. The relative broad range of size and complexity in each region detected by flow cytometry was corroborated by light microscopy. The cells included in the R1 corresponded to granulocytes (Figures 1B-1D). Different shapes and morphologies were observed in fresh samples after cell attachment to the glass surface (Figure 1C). Granulocytes were the largest cells, showing a low nucleus-cytoplasm ratio. Those cells were composed by an endoplasm with high number of granules and an extended hyaline ectoplasm (Figure 1C). The cells included into the R2 matched with hyalinocytes (Figures 1E-1G). Hyalinocytes were the smallest cells and were characterized by the lack of endoplasm and the high nucleus-

cytoplasm ratio (Figure 1F). The cells included in the R3 were characterized as intermediate cells (Figures 1H-1J). They presented a high nucleus-cytoplasm ratio, with the cytoplasm mainly composed by an endoplasmatic region with the ectoplasm reduced to a small striped region surrounding the endoplasm (Figure 1I).

3.2. Functional characterization of *R. decussatus* haemocytes

3.2.1. Phagocytosis assay

The cell populations showed different ability to phagocyte the FITC-labelled particles (latex beads, zymosan and *E. coli*). The phagocytosis was detected as an increase of the fluorescence levels registered in the FL1-H channel (Figure 2A). The phagocytic levels of cells treated with latex beads were significantly higher than the levels registered after zymosan or *E. coli* treatment regardless of the cell population (Figure 2B). The easiest phagocitable particles were the latex beads, following by the zymosan and the *E.coli*. Moreover, after two hours of latex exposition, the 40% of the cells in R1 ingested at least one particle in contrast with 10% of R3 cells and 5% of R2 cells (Figure 2B). Phagocytosis levels registered in the R1 after treatment with latex beads or zymosan particles were significantly higher than those of the cells included in the R2 or R3 (Figure 2B). Haemocyte samples were also observed by microscopy after phagocytosis. Although the internalization of the particles was corroborated in all cell types (Figure 2C-2G), the granulocytes were the cells with the highest phagocytosis rate, showing also the highest number of particles into the cytoplasm (Figure 2D and 2E).

3.2.2. Respiratory burst assay

The production of reactive oxygen species after the stimulation with zymosan was detected in the FL1-H channel as an increase in the mean fluorescence value. Moreover a decrease of this value was recorded in the same samples treated with SOD (Figure 3A). The clam respiratory burst activity was low and only 8 out the 24 analyzed clams (33 %) produce detectable levels of oxygen radicals in the three cell populations at the same time. The percentage of active clams increased to 83.3 %, 41.6 % and 62.5 % when only one cell population was considered (R1, R2 and R3, respectively). In all populations the stimulation with zymosan induced significant changes in the mean fluorescence values compared to the levels registered in control samples (Figure 3B).

There were variations in the fluorescence values detected in each region from one clam to another (Figure 3C). Granulocytes (R1 cells) showed the highest and the hyalinocytes showed the lowest values of ROS production. The average fluorescence levels registered in R1, R2 and R3 cells were 131, 5 and 34 respectively (Figure 3C).

3.3. *In vitro* stimulation of *R. decussatus* haemocytes

3.3.1. Effect of the stimulants on the cell viability

The exposure of the haemocytes to the different treatments induced significant changes in the cell viability (Figure 4). The analysis of the whole population revealed that the samples treated with LPS showed significant lower levels of damaged cells than those registered in the control group 1 h and 3 h after the treatment. Also those values were significantly lower than the levels registered in samples treated with dead or live bacteria regardless of the sampling point (Figure 4A). The treatment with dead bacteria and live *V. splendidus* reduced the cell viability in all the sampling points evidenced as a significant increase in the number of stained cells (PI+) in comparison with controls.

The response of the R1 cells to the different treatments was quite similar (Figure 4B) except for the LPS treatment. A significant 2-fold increase in the number of PI+ cells was detected in samples treated with dead bacteria in all sampling points. Live bacteria also induced a significant increase in the number of damaged cells, reaching a 2-fold change at 6 h. This reduction of the cell viability increased between 1 h and 6 h.

3.3.2. Effect of the stimulants on the gene expression profile

Changes in gene expression profiles following stimulation of haemocytes with LPS, dead bacteria or *V. splendidus* infection are summarized in Figure 5. The expression of the DAD-1 gene was significantly increased at 3 h post-treatment in haemocytes stimulated with LPS, when it reached the maximum expression value (2-fold increase). At 6 h the values decreased to control levels. The infection with *V. splendidus* only induced a significant decrease of the DAD-1 gene 1 h after infection. No significant changes were registered at 3 h and 6 h post infection (Figure 5A). The expression of the thrombin gene increased at 6 h in samples treated with LPS or dead bacteria, although the values were not significantly different compared to the control. Infection with *V. splendidus* did not induce any significant change (Figure 5B). Prosaposin expression was modulated in samples treated with dead bacteria, reaching significant maximum values (2-fold increase) at 6h, meanwhile samples treated with LPS decreased to control values. The infection with *V. splendidus* did not induce any significant change (Figure 5C). Moreover, a 20-fold increase in IAP expression was recorded in haemocytes treated with dead bacteria 6 h post stimulation (Figure 5D). The factor B gene and the complement component C3 gene showed similar kinetics. No changes in gene expression were detected in samples infected with *V. splendidus.* Haemocytes stimulated with LPS or dead bacteria showed an up-regulation of both

genes 1 h and 6 h post stimulation although only the expression levels of factor B registered at 1 h were significantly different compared to the control (Figures 5E and 5F).

4. Discussion

The two main bivalve haemocyte types, granulocytes and hyalinocytes, were observed in *R. decussatus* haemolymph. They differed in size, number of granules and nucleus:cytoplasm ratio being granulocytes larger and with higher number of granules and cytoplasm ratio with the nucleus. It was also detected a third group of haemocytes with intermediate values of size and granularity that we have named intermediate cells. In the clams *M. mercenaria*, *Meretrix lusoria* and the oyster *C. virginica* were also described a group of cells, smaller and less complex than granulocytes, that have been denominated fibrocytes, small granulocytes or intermediate cells [5, 11, 12, 29, 66]. It has been postulated that these intermediate cells could actually be degranulated granulocytes in the final step of their vital cycle [11, 67], although some authors proposes that they are active cells [68]. The identification of the two main subpopulations (granulocytes and hyalinocytes) has been described in numerous bivalves [8, 9, 14-17]. Granulocytes are the main immune-related subpopulation, with higher phagocytic ability and ROS production than hyalinocytes [69, 70]. Moreover, granulocytes are related to encapsulation process. Hyalinocytes seem to be more important in hemocyte aggregation process [18].

However, we have observed three different subpopulations based on their relative size and complexity by flow cytometry. Methodology, endogenous and exogenous factors, like age and pollution, and the high inter-individual variability

observed in bivalves could have influence in the results [12, 14, 15, 71-73]. To minimize this discrepancy, several techniques of cell identification can be used simultaneously. Among them, the physical separation by flow cytometry (cell sorting), was postulated as a useful method to distinguish between subpopulations in bivalves [37]. We have confirmed that *R. decussatus* haemocyte types observed by light microscopy corresponded with the subpopulations selected in the flow cytometry sorting process. Flow cytometry has major advantages when compare to traditional methods such as microscopy or density gradients since is possible to compare simultaneously several parameters at cellular level, as size and complexity, phagocytic activity and ROS production, leaving the possibility of use of specific markers [5]. In particular, flow cytometry has been shown to be very sensitive in the detection of respiratory burst activity compared with the classic luminol-dependent chemiluminiscence or NBT reduction, and is focused in individual cells more than in total populations [5, 39].

Regarding phagocytosis and ROS production, we have found that granulocytes were more active phagocytising foreign particles. However, both granulocytes and hyalinocytes were able to phagocyte particles in concordance with previous studies conducted on *M. galloprovincialis*, *Cerastoderma glaucum*, *M. lusoria*, *M. mercenaria* and *A. irradians* [10, 15, 23, 28, 29]. On the other hand, in other species like *Tridacna crocea*, *Cerastoderma edule* and *R. philippinarum* (=*Tapes semidecussatus*) phagocytosis in hyalinocytes has not been detected [10, 18, 73, 74]. The discrepancy could be related with the sensitivity of the method, with the lack of antibodies for the characterization of the different populations and also with the seasonal and inter-individual variability. Related with the phagocytosis process, high reactive oxygen radicals are released during the oxidative burst that can be detected by luminol or

lucigenin dependent chemiluminescence, the NBT reduction and the cytochrome-c reduction [6, 21, 32, 34, 45, 75, 76]. There is some controversy about the ability of clam species to produce ROS [77]. In *Siliqua patula* and *Tapes philippinarum* it has been detected ROS by lucigenin dependent chemiluminescence and NBT reduction. However, these probes failed in detecting ROS in the clams *R. decussatus*, *M. mercenaria*, *M. arenaria* and *S. plana* [45, 47]. The extremely low amount of radicals released in clam species comparing to other bivalve species could explain the lower detection of ROS in clams when they are analysed by classical methodologies. As it has been postulated before, the detection of ROS is dependent of the method utilised and its sensitivity, the stimulus used and the composition of the medium to maintain the haemocytes [42, 77, 78]. The use of a more sensitive methodology such as flow cytometry, allowed the detection of ROS release in *C. gigas* and *R. philippinarum* [43, 44] and the detection of H_2O_2 production in *M. mercenaria* [42]. Regarding *R. decussatus*, several attempts without success have been previously made to detect oxidative burst by applying the commonly used methodologies [46, 47]. However we have confirmed by flow cytometry that *R. decussatus* haemocytes release oxidative radicals. Our results showed that granulocytes, as occurs in *C. virginica* [5], were the haemocytes that release more oxygen radicals. In hyalinocytes we have also detected respiratory burst although at a very low level. In intermediate cells we have found moderate values compared with granulocytes and hyalinocytes. To test the specificity of the reaction we have used the superoxide dismutase enzyme (SOD) [6]. We have not observed a complete inhibition due to the triggering of reactive oxidative species that are not specifically inhibited by the SOD, suggesting that several oxygen species forms the oxidative defence of *R. decussatus*. This suggests that DCFH oxidation detection by

418 flow cytometry is a good system to detect the low levels of oxygen radicals released by
419 *R. decussatus*.

420 The infection of haemocytes with live or dead bacteria induced an expected
421 decrease in the cell viability as it was previously described [55]. The effect of the
422 bacterial infection on the gene expression profile was assayed by analyzing the changes
423 in genes related to stress and apoptosis. Candidate genes related to complement system
424 and apoptosis were selected from previous works because they showed a modulated
425 expression after protozoan infection and bacterial stimulation of *R. decussatus* [62-64].
426 The DAD-1 gene has been characterized to play roles in the apoptotic process [79] and
427 is over-expressed after different stimuli such as tissue damages, infections and stress
428 induced by environmental changes [80-82]. The regulatory function of this protein to
429 inhibit apoptosis has also been described in scallops [82]. The stimulation of the
430 haemocytes with LPS induced a significant up-regulation of this gene at 3 h when the
431 maximum levels of cell viability were registered in R1 and also in the whole cell
432 population. Interestingly, the infection with *V. splendidus* induced a significant down-
433 regulation of this gene 1 h after infection, suggesting that apoptosis could be involved in
434 the pathogenesis associated with the bacterial infection as it was described in other
435 bacterial models [81, 83]. Gagnaire et al. [84] also reported that *V. splendidus* induced a
436 down-regulation of SOD, that is a protective protein against reactive oxygen species, in
437 *C. gigas*. The apoptotic process was also analyzed by measuring the expression levels of
438 the IAP gene and the prosaposin gene. The prosaposin gene is not directly involved in
439 the apoptotic process, although the generation of bioactive ceramides are involved in the
440 regulation of apoptosis by the activation of proapoptotic caspases [85]. The infection
441 with *V. splendidus* did not modulate the expression levels of both genes and only the
442 treatment with LPS induced high expression levels of IAPs and prosaposin gene (25 and

2-fold changes respectively). The high expression levels of IAPs in LPS stimulated cells could be related with the increase on the cell viability. The serine-protease enzymes are involved in different immune processes such as coagulation, phagocytosis, activation of the complement cascade [86] or activation of the prophenoloxidase system that has been described in different bivalve molluscs [87-90]. The thrombin gene and the factor B gene contained a 3´serine-protease domain [63]. In vertebrates the factor B induces the activation of the C3 by the alternative pathway and its expression is modulated by different stimuli [91, 92]. Both genes were up-regulated after the stimulation with LPS and dead bacteria. Cathepsin L, a similar proteinase located in lysosomes has been previously observed up-regulated 24h after a *V. splendidus*-related infection in *C.gigas* [84]. The biological process that is activated after the expression of this serine-protease similar to thrombin has not been already described in bivalves, but it could be a mechanism similar to the coagulation pathway described in *Tachypleus tridentatus* after LPS stimulation [93]. It could be possible that the release of extracellular products by *V. splendidus* can degrade the complement proteins as it was previously described [94].

In conclusion, the flow cytometry is a suitable technique to study the morphology of *R. decussatus* haemocytes and also to measure some immune parameters such as the phagocytosis and the production of oxygen radicals. The infection with *V. splendidus* induced a decrease in the cell viability. Opposite to the results obtained in samples stimulated with LPS or dead bacteria, *V. splendidus* did not modify the expression levels of genes related with stress, apoptosis and serine-protease activity suggesting that the bacterial extracellular products could modulate the immune response.

Acknowledgements

Maria Prado wishes to acknowledge CSIC for her I3P fellowship. This work has been funded by the Spanish Ministerio de Ciencia e Innovación (MICINN) (AGL2008-05111).

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Tables

Table 1: Sequences of specific primers used for qPCR assays.

Target	Sequence Forward	Sequence Reverse
DAD-1	GCTATTGTGCATTGGTTGGA	AATGCTCTTTCTGGGCTGAT
Thrombin	CGTTTGTTGTTCGACATCCT	ATGATCCTTGTTCCGCTTTC
Prosaposin	TGCATTTTCTTTTGCTTTCG	TGTTTGGTAGCCCCACATT
IAP	CAGAGGAGTTGCAGTCGGTA	TACATCTGCCCTTTTGTCCA
Bf	GACAGATGCTGAGGAAACG	GTGCGGTGTTGAGGCTATTT
C3	CGGCAAAGGCTTTATTGTGT	TGAGTGCAGTGCCTATCTGG
Actin	CGACTCTGGAGATGGTGTCA	ATGAGTAAGTGTTGGTGGCG

748 **Figure legend**

749

750 **Figure 1. (A)** Density plot distribution of haemocyte populations in relative size (FSC-
751 H) and complexity (SSC-H) by flow cytometry. Granulocytes, hyalinocytes and
752 intermediate cells were included in the regions R1, R2 and R3, respectively. **(B)**
753 Microphotograph of ethanol fixed and stained granulocyte. **(C)** Fresh granulocyte under
754 Nomarski DIC objective. **(D)** Fresh granulocyte after sorting. **(E)** Microphotograph of
755 ethanol fixed and stained hyalinocyte. **(F)** Fresh hyalinocyte under Nomarski DIC
756 objective. **(G)** Fresh hyalinocyte after sorting. **(H)** Microphotograph of ethanol fixed
757 and stained intermediate cell. **(I)** Fresh intermediate cell under Nomarski DIC objective.
758 **(J)** Fresh intermediate cell after sorting. (n) nucleus, (hy) hyaloplasm, (en) endoplasm,
759 (ec) ectoplasm. Scale bar 10 µm.

760

761 **Figure 2**: **(A)** Histogram of fluorescence registered in the FL1-H channel in R1 cells
762 (granulocytes) treated with latex beads. **(B)** Percentage of granulocytes (R1),
763 hyalinocytes (R2) and intermediate cells (R3) that ingested at least one fluorescent
764 particle (*E. coli*, latex beads or zymosan). The bars represent the mean ± standard
765 deviation of 16 samples in each treatment. (a) Significant differences in each region
766 regarding to the levels obtained in haemocytes treated with latex beads. (b) Significant
767 differences within each treatment regarding to the level recorded in R1. **(C)**
768 Photomicrography of a control granulocyte. **(D)** Granulocyte ingested several latex
769 beads after 2 hours of incubation. **(E)** Granulocyte engulfed zymosan particles. **(F)**
770 Intermediate cell engulfed one zymosan particle. **(G)** Hyalinocyte engulfed one latex
771 bead. Scale bar 10 µm.

772

Figure 3: (A) Histogram representing the mean fluorescence of a representative sample of granulocytes (R1 cells) stimulated with zymosan and treated with zymosan plus SOD at the same time. **(B)** Results represent the mean ± SD of the fluorescence values recorded in responsive animals. Results from 13, 10 and 15 animals were considered in the R1, R2 and R3, respectively. The y axis showed relative units of fluorescence (mean fluorescence values). (*) Significant differences regarding to the levels obtained controls. **(C)** Response of eight representative clams exposed to FSW (control), zymosan and zymosan plus SOD. R1 (granulocytes). R2 (hyalinocytes). R3 (intermediate cells).

Figure 4: Viability of haemocytes treated with LPS, dead bacterial mixture and *V. splendidus* after 1 h, 3 h and 6 h post treatment. (A) Results obtained in the whole population. (B) Results obtained in R1. Significant differences ($p<0.05$) regarding to the levels obtained in control, LPS and dead bacteria treated samples, were indicated with a, b and c, respectively.

Figure 5: Modulation of DAD-1 (A), thrombin (B), prosaposin (C), IAP (D), factor B (E) and complement component C3 (F) in haemocytes stimulated with LPS, *V. splendidus* and dead bacterial mixture 1 h, 3 h and 6 h post treatment. Results represent the mean ± SD of 4 experimental haemocyte pools. Data were analyzed using the Student's t-test. a, b and c indicate significant differences ($p<0.05$) of each gene in haemocytes stimulated with LPS, *V. splendidus* and the bacterial mixture, respectively compared to the control samples.

Figure 1

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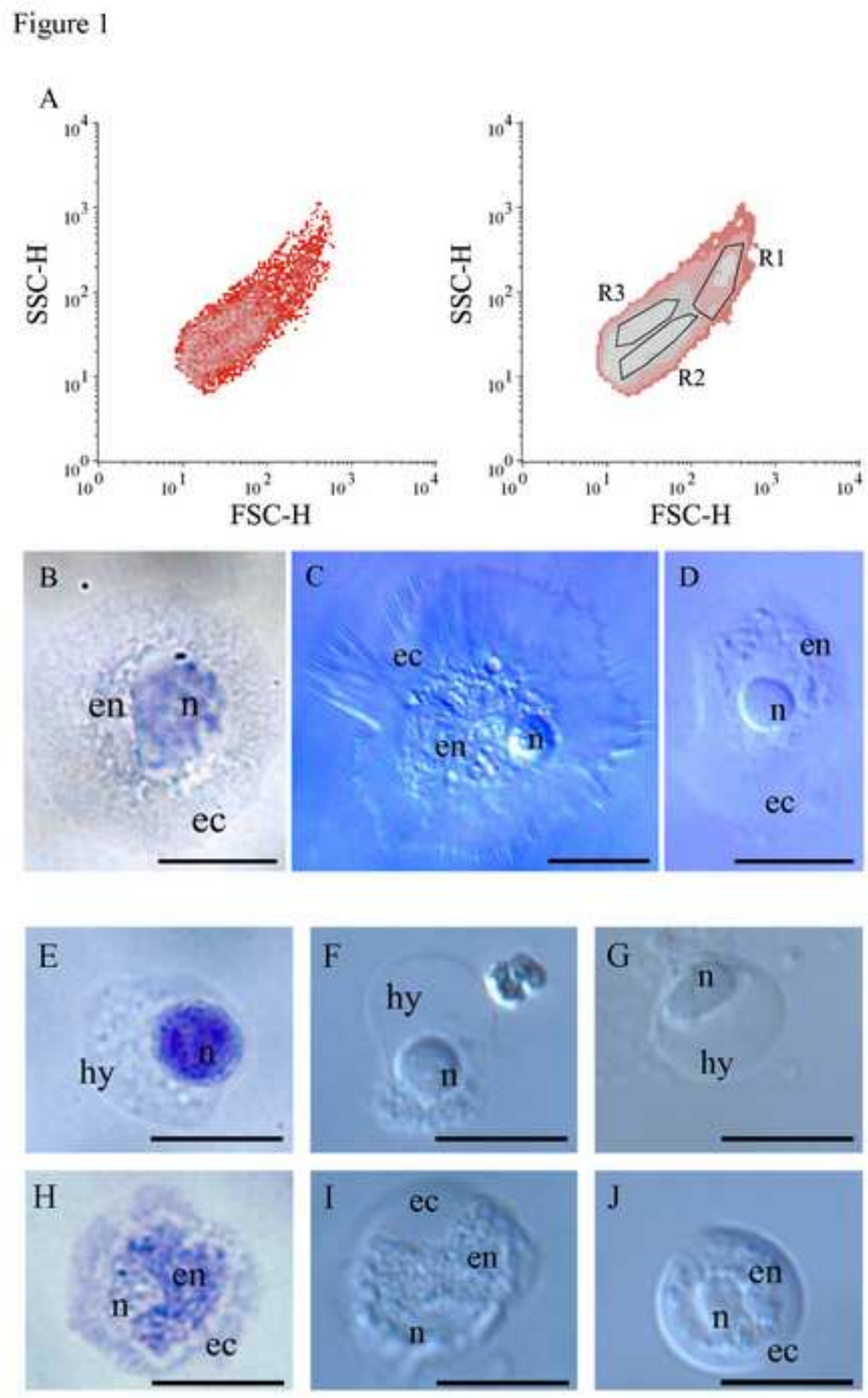

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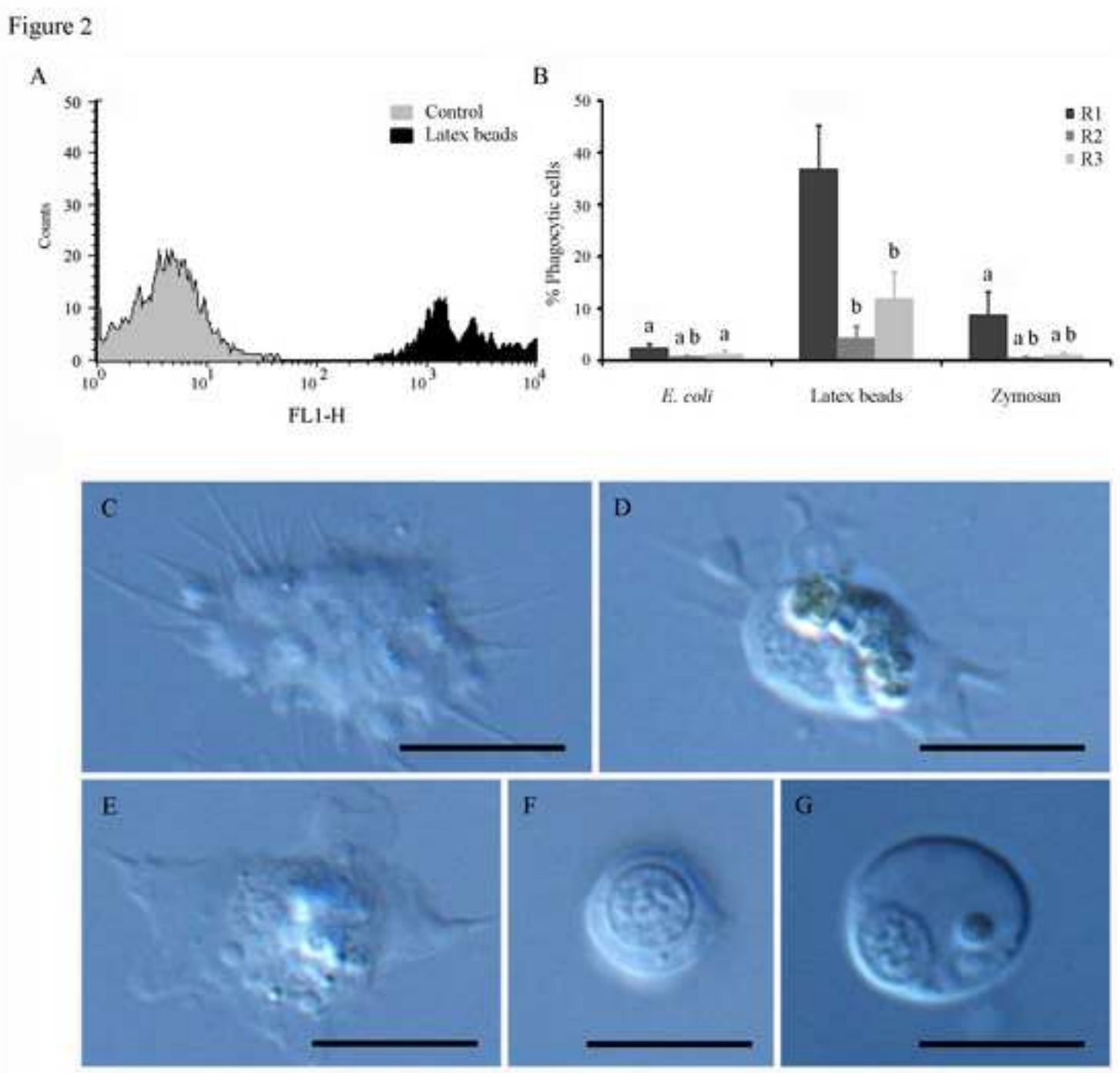

Figure 3

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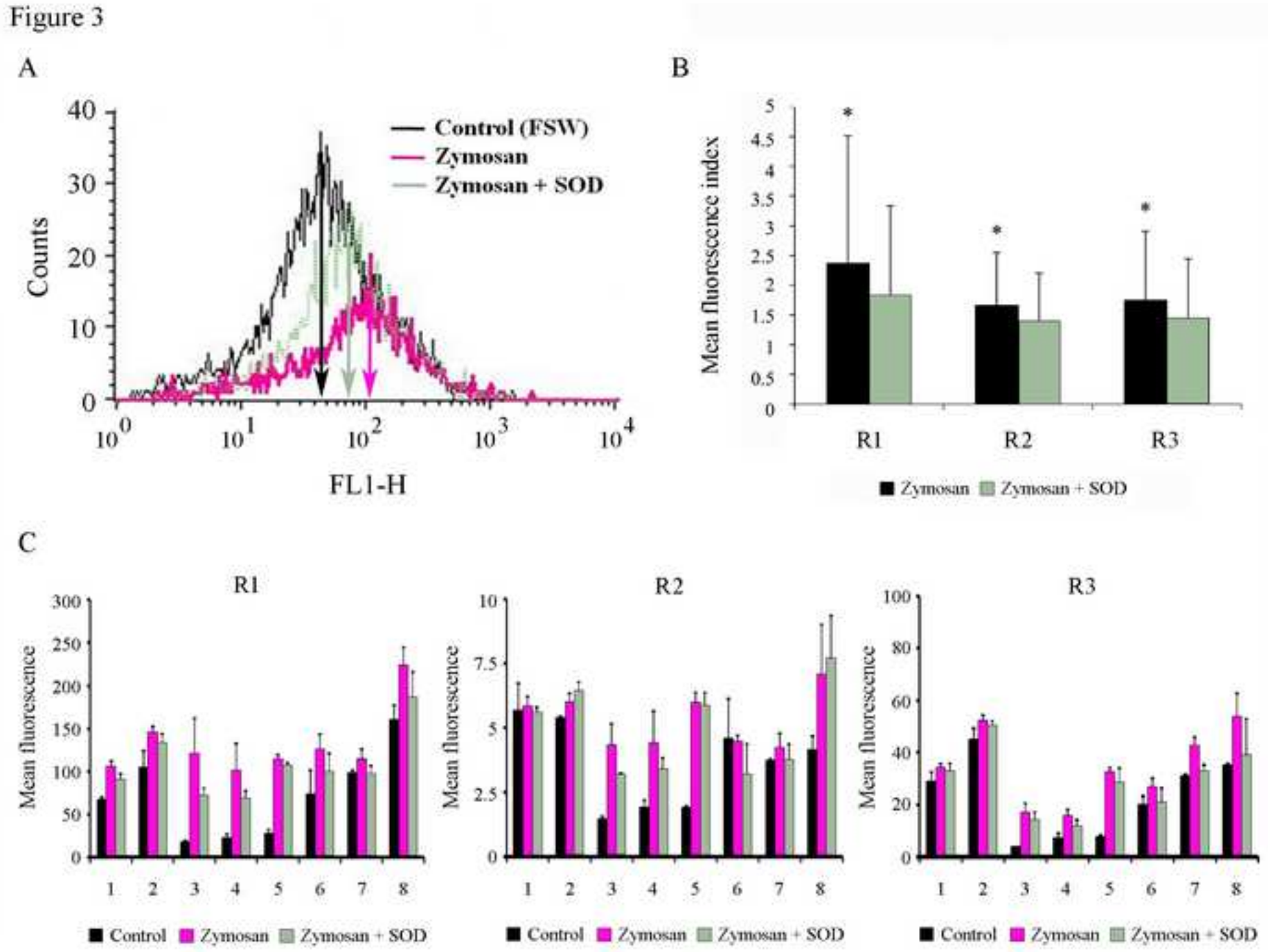

Figure 4
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Figure 4

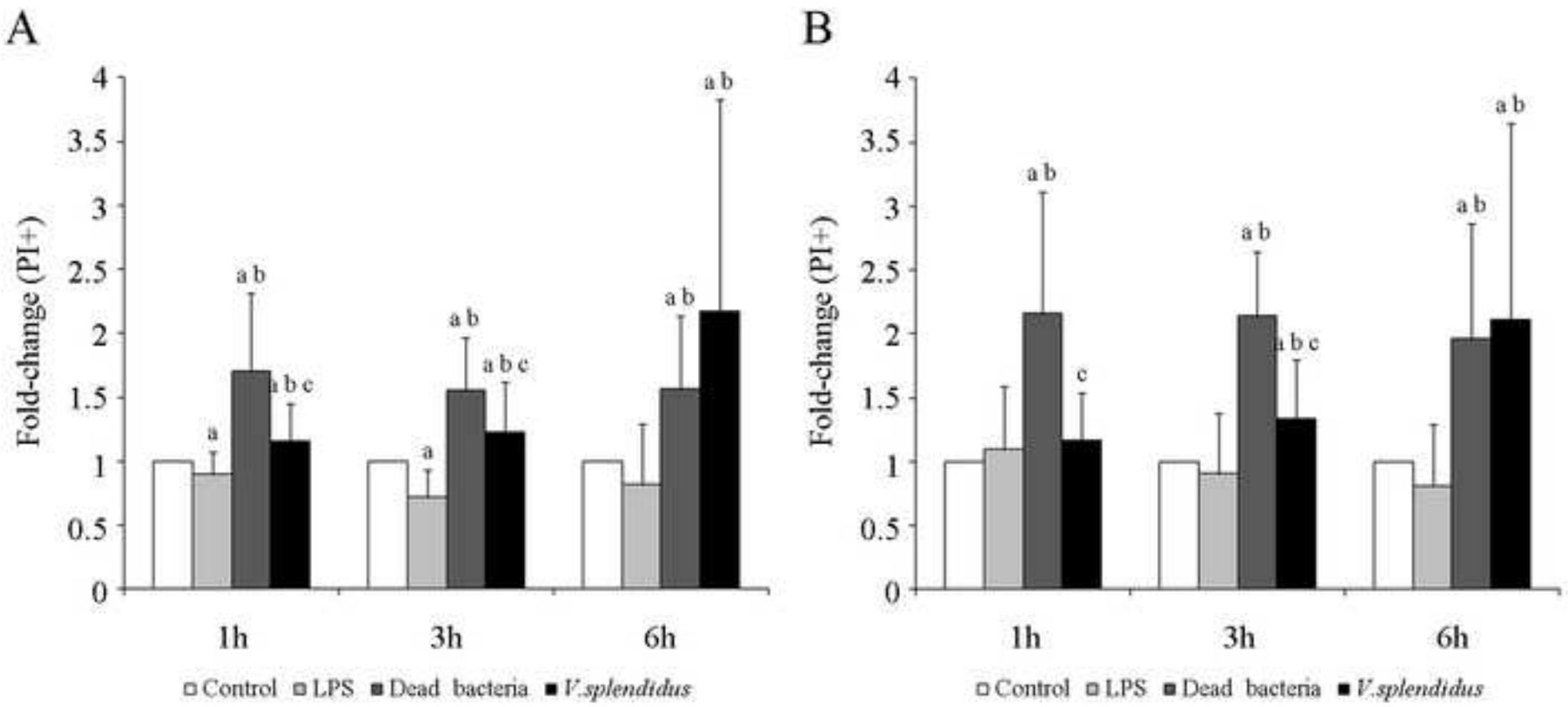

Figure 5

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