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Effects of extracellular products from the pathogenic Vibrio aestuarianus strain 01/32 on lethality and cellular immune responses of the oyster Crassostrea gigas

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Abstract: Vibrio aestuarianus strain 01/32 was previously shown to be pathogenic to Crassostrea gigas juveniles. To investigate virulence mechanisms of this pathogen, we studied the toxicity to oysters of its extracellular products (ECPs). ECPs displayed lethality to animals, with a LD50 value of 3.3 µg/g body weight. To determine the oyster cellular immune responses induced by these ECPs, we further examined in vitro their effects on C. gigas hemocytes, using flow cytometric-based hemocyte assays. Treatment of hemolymph with ECPs caused a significant inhibition of hemocyte phagocytosis and adhesive capabilities. In contrast, the pathway of reactive oxygen species production was enhanced by higher ECP concentrations. Exposure of hemocytes to live bacteria induced no changes in hemocyte parameters. Together, these results suggest that V. aestuarianus strain 01/32 secretes one or more factors which may play an important role in the pathogenicity of this microorganism, and which display immunosuppressant activities on hemocyte functions.

Keywords: Bivalve immunity; Flow-cytometry; Phagocytosis; Adhesion; ROS production; Crassostrea gigas; ECPs; Vibrio aestuarianus

1. Introduction

Under intensive culture conditions, marine species are exposed to various stressors, including bacterial pathogens. Among possible sources of bacterial infections, *Vibrio* spp. are often associated with diseases in larval and adult stages of bivalves [1-5]. In France, since 1991, high mortality rates of *Crassostrea gigas* spat (60 to 100%) have been reported during summer, both in the field and in hatcheries, and are of major concern to oyster farmers [6]. Several hypotheses concerning the etiology of these summer mortalities have been explored, including the involvement of potentially-pathogenic agents. To date, a herpes-like virus [7, 8] and several *Vibrio splendidus* strains [9-11] have been detected in oyster spat populations experiencing summer mortalities. Although these infectious agents were demonstrated to be pathogenic to *C. gigas* spat under experimental conditions, none could be systematically associated with the onset of these epizootics in the field [11]. One can hypothesize, therefore, that mortality could be the result of complex interactions between the physiological and/or genetic status of the host, numerous environmental factors, and one or more infectious agents acting more as opportunists than pathogens [6, 11].

To explore this hypothesis of opportunistic infection of stressed oysters, a bacterial strain was isolated from moribund *C. gigas* juveniles, sampled during a summer mortality outbreak in an experimental hatchery. Hemolymph of moribund animals contained a consistently-high load of bacteria, revealing septicemia, and was infected predominantly with a *V. aestuarianus* strain isolated and given the strain designation 01/32. Its pathogenicity was assessed by experimental challenge, resulting in high *C. gigas* mortality rates (60-90 %) (M. Garnier, pers. comm.). Accordingly, this *Vibrio* strain was judged to be a suitable candidate for the study of bacterium-host interactions with *C. gigas*.

Although some manifestations of bacterial infections in oysters have been described (see references [12, 13] for a review), little is known about bacteria-induced host tissue alterations, as well as immune responses, compared to the studies on the pathogenic processes for vertebrates and plant pathogens. During the process of infection, bacteria are confronted by oyster cellular immune defenses, specifically by immuno-competent cells referred to collectively as hemocytes. These cells, which circulate within the oyster open vascular system and across all epithelial boundaries, constitute the main line of host internal defenses against parasites and pathogens. Some disagreements remain concerning the number of hemocyte sub-populations in bivalves, but attempts at developing a uniform classification of bivalve hemocytes have resulted in recognition of three major cell types: granulocytes, hyalinocytes and agranulocytes [14-16]. There is general agreement that granulocytes function most actively in the phagocytosis of bacteria. This activity is usually accompanied by the production of reactive oxygen species (ROS), also known as “respiratory burst”,

which display strong microbicidal activities. Hyalinocytes are less phagocytic than granulocytes and their main defense function appears to be wound repair (see references [16, 17] for a review). However, despite numerous histological and ultrastructural observations, the precise roles of these basic cell types, the mechanisms by which they function, and their susceptibility to bacteria remain poorly understood.

Previous studies demonstrated that bacteria possess different survival capabilities in bivalve tissues and hemolymph [18, 19]. This difference in susceptibility may depend on several factors, which may include avoiding contact with phagocytic hemocytes, inhibition of phagocytic engulfment, survival inside phagocytic cells, and the production of components that kill or damage hemocytes [20]. For example, *V. tapetis*, the causative agent of brown ring disease (BRD) in the clam *Ruditapes philippinarum*, avoids phagocytic engulfment by impairing hemocyte adhesion properties [21]. Some bacteria possess the capacity to inhibit the ROS production associated with phagocytosis. Bramble *et al.* [22] demonstrated, using chemiluminescence (CL) assays, that the bacterium *Listonella anguillarum* expresses an antioxidant enzyme, suppressing the production of ROS by *C. virginica* hemocytes. In addition, *Vibrio* sp. strain S322 was shown by flow-cytometric measurements to inhibit the CL response of scallop and oyster hemocytes [23, 24] and the ROS production of *C. gigas* hemocytes [25]. An understanding of interactions between pathogenic bacteria and oyster hemocytes is, therefore, of particular interest to elucidate mechanisms responsible for bacterial persistence in oyster fluids and tissues.

In the past, the virulence of pathogenic *Vibrio* spp. isolated from oysters was shown to be related to their ability to produce extracellular products (ECPs) [1, 2]. According to Maeda *et al.* [26], these ECPs, mostly consisting of proteases, could facilitate the propagation of the bacteria by causing extensive host tissue damage, thereby degrading host proteins to provide readily-available nutriments for bacterial growth. Furthermore, ECPs could also counteract the host defense system by degrading immunoglobulins and components of the complement system, for example [26]. Mechanisms involved in the pathogenicity of *V. aestuarianus* 01/32 to oysters are currently unknown, and the contribution of extracellular products to the pathogenesis of this strain needs to be ascertained. This study was aimed at investigating the overall hypothesis that *V. aestuarianus* 01/32 ECPs impair immunity and survival of oysters. Specifically, the present work was designed to determine the toxicity of *V. aestuarianus* 01/32 ECPs *in vivo* and to evaluate *in vitro* the oyster cellular-immune responses after exposure to these extracellular products.

2. Materials and methods

2.1 Oysters

One-year-old oysters *C. gigas* (mean weight 5 ± 1g) were provided by a French commercial hatchery (SATMAR, Lannilis, France) and grown out in Aber Benoît (Finistère, France). Animals were acclimated for two weeks in the laboratory (IFREMER, Plouzané, France) in a flow-through seawater system at ambient temperature (13-15°C) and salinity (33-35 ‰), and were fed a mixture of two marine microalgae: *Isochrysis aff. galbana* clone T-Iso (also termed Tahitian *Isochrysis*) and *Pavlova lutheri*.

2.2 Preparation of V. aestuarianus 01/32 bacterial suspension

A *Vibrio* sp. was previously isolated from 18 month-old oysters (*C. gigas*) during a mortality outbreak occurring in June 2001 at the IFREMER experimental hatchery (Finistère, France). This *Vibrio* sp. was identified as *V. aestuarianus* by phenotypic (API 20E and 50 CH identification kits, BioMérieux France) and genotypic methods, based on phylogenetic analyses of 16S ribosomal DNA (GenBank accession number AJ845023) and gyrase B subunit (GenBank accession number AJ582818) genes (M. Garnier, pers. comm.). The strain was given the designation 01/32. *V. aestuarianus* strain 01/32 was routinely cultured in Marine Broth (MB, Difco) (20°C, 20-h, shaker table at 200-rpm). Bacterial cells were harvested by centrifugation (1,500XG, 10-min), washed twice with filtered (0.22-µm) sterile seawater (FSSW) and re-suspended in FSSW. Bacterial cell concentration was estimated using a cell number-optical density (550-nm) relationship previously established.

2.3 Preparation of V. aestuarianus 01/32 extracellular products

Bacterial ECPs were produced by the cellophane overlay method as described by Liu *et al.* [27]. Tubes containing 5-mL of MB were inoculated with one bacterial colony from a 24-h Marine Agar (MA, Difco) culture of *V. aestuarianus* strain 01/32 and incubated at 20°C for 18-h. A volume of 2-mL of this culture was transferred onto a sterile cellophane film placed on the surface of each MA plate. After incubation at 20°C for 48-h, the cellophane overlay was transferred to an empty Petri dish. Cells were washed off the cellophane film using 4-mL of cold FSSW and removed by centrifugation at 10,000XG and 4°C for 30-min. The supernatant containing the ECPs was sterilized by filtration (0.22-µm) and stored at –80°C until use. The protein concentration of the ECPs was measured by the method of Bradford

[28], with bovine serum albumin (Sigma) as the standard.

2.4 ECP toxicity testing and LD_{50} determination

The role of ECPs in the pathogenicity of *V. aestuarianus* 01/32 to *C. gigas* was evaluated, and the 50% lethal dose (LD_{50}) was determined. To anaesthetize oysters and open the valves, animals were first kept for 3-h in a $MgCl_2$ bath at a final concentration of 50-g.L^{-1} (2/3 v/v seawater/freshwater), according to the method of Gay *et al.* [29]. A volume of 0.2mL of the ECP solution was then injected into the adductor muscle of each oyster. Four different doses of ECPs (1.25, 2.5, 5 and 10 µg protein/g body weight) were tested, and each dose was injected into 10 oysters. As controls, one group of 10 oysters was injected with FSSW, and an additional group was not injected. Immediately after injection, oysters were transferred to aquariums containing aerated filtered seawater at ambient temperature (19 ± 1°C) and kept under static conditions. Observations were made twice a day, and mortalities were recorded for 7-days after injection. Animals were considered as dead when the valves did not shut anymore and the mantle did not react after stimulation by prick. The experiment was carried out in duplicate and LD_{50} values were calculated by the statistical method of Reed & Müench [30].

2.5 Hemolymph sampling and hemocyte parameters

A small notch was carved in the dorsal side of the shell, adjacent to the adductor muscle, of unchallenged oysters. Hemolymph samples were withdrawn from the adductor muscle using a 1-mL plastic syringe fitted with a 25-gauge needle, and samples were stored individually in micro-tubes held on ice, to minimize cell clumping. Individual samples were observed under the optical microscope to control the quality of the hemolymph used subsequently and to prevent gut-bacterial or gamete contamination. Hemolymph was then filtered through 80-µm mesh, and five individual samples were pooled. Four pools were prepared for each treatment. This operation was repeated for each of the experiments performed.

Phagocytosis of fluorescent beads

The phagocytosis assay was adapted from the method of Delaporte *et al.* [31], using fluorescent, latex beads (Fluoresbrite, YG Microspheres, 2 µm, Polysciences). 300-µL sub-samples of each hemolymph pool were distributed into 5-mL polystyrene tubes (Falcon®) and maintained on ice. ECPs or bacteria (300-µL) were added according to

experimental design described below. A control was included by adding FSSW (300-µL) to one sub-sample of each pool. Each sub-sample was subsequently incubated with 30-µL of fluorescent bead working solution (2% of the commercial solution in FSSW) at 18°C for 60-min. Tubes were then analyzed on the flow cytometer. As the number of beads engulfed in hemocytes differed, several fluorescence peaks were visible on the cytogram, corresponding to the number of beads associated with the hemocytes (Fig. 1). Results of phagocytosis were expressed as the percentage of hemocytes containing three beads or more, according to Delaporte *et al.* [31] and Hégaret *et al.* [32].

Adhesive capacity

To estimate hemocyte adhesion capacity, 100-µL sub-samples of each hemolymph pool were distributed into 24-well microplates maintained on ice. Each sub-sample received a 100-µl volume of ECPs or bacteria, or 100-µL of FSSW as a control. After three hours of incubation at 18°C, cells were fixed by addition of 200-µL of a 6% formalin solution in FSSW. Concentration of non-adherent cells was established according to the method described by Delaporte *et al.* [31]. For each condition, the percentage of adhering hemocytes was calculated relatively to the initial total hemocyte count of the tested pool.

Reactive oxygen species (ROS) production

The method using 2'7'-dichlorofluorescin diacetate (DCFH-DA, Sigma), previously described in mammals by Bass *et al.* [33], was adapted to *C. gigas* by Lambert *et al.* [25] and to *C. virginica* by Hégaret *et al.* [34] was used. Briefly, intracellular DCFH oxidation is quantitatively related to the oxidative metabolism of hemocytes and primarily mediated by H_2O_2 but also by other ROSs, such as superoxide anion, peroxyl radical and peroxynitrite anion [35]. DCF production results in green fluorescence, which is measured on the FL1 detector of the flow cytometer.

A 150-µL sub-sample of hemolymph from each pool was distributed into a 5-mL polystyrene tube (Falcon®), diluted (+ 450-µL) with FSSW and maintained on ice. Each sub-sample received a 300-µl volume of ECPs or bacteria, or 300-µL of FSSW as a control. DCFH-DA working solution was finally added in each tube maintained on ice to yield a final concentration of 10-µM. Tubes were then incubated for 60 and 120-min at room temperature in the dark. The DCF green fluorescence level was evaluated for the three hemocyte sub-populations: agranulocytes, hyalinocytes and granulocytes, distinguished according to their relative size (forward scatter, FSC) and complexity (side scatter, SSC) (Fig. 2). Small agranulocytes showing very low ROS production; values for hyalinocytes and granulocytes only are presented here. Results are given for both hemocyte sub-populations as mean of fluorescence in FL1 arbitrary units.

2.6 Hemocyte responses to different concentrations of ECPs

A first set of experiments was conducted *in vitro* to determine the effect of increasing concentrations of bacterial ECPs on several hemocyte parameters. ECPs (300-µL) were added to obtain final concentrations of 2, 4, 8, 16, 32, 64 and 128-µg.mL^{-1}. Measurements of phagocytosis, adhesion, and ROS production by hemocytes were processed as described above.

2.7 Minimal inhibitory concentration of ECPs

A second set of experiments was performed to determine the minimal inhibitory concentration of ECPs on hemocyte phagocytic and adhesive activities. Hemolymph sub-samples were treated following the methods described above, except that ECPs were added at lower concentrations than previously tested, ranging from 0.1 to 2-µg.mL^{-1} (0.1, 0.5, 1 and 2-µg.mL^{-1}).

2.8 Comparison of V. aestuarianus 01/32 live cells and ECPs on hemocyte responses

A third set of experiments was performed to evaluate the influence of live bacterial cells on phagocytic, adhesive and ROS-producing capacities of hemocytes, in comparison to low (2-µg.mL^{-1}) and intermediate (32-µg.mL^{-1}) doses of ECPs. To adjust the bacteria/hemocyte ratio, the hemocyte concentration of pooled hemolymph was evaluated according to Lambert *et al.* [25]. The *V. aestuarianus* 01/32 bacterial suspension was prepared so as to obtain a ratio of 50 bacteria per hemocyte (final concentration). Thereafter, the same experimental procedures as described above were used. Incubation time and measurement remained the same as those previously established.

2.9 Statistical analysis

Significant differences between treatments during each assay were tested by one-way analysis of variance (ANOVA), or Kruskal-Wallis test in case of heterogeneity of variances, using Statgraphics Plus 4.1 software. Data collected as percentages were transformed (arcsine of the square root) before analysis but are presented in figures as untransformed percentage values. The method used to discriminate among the means was Fisher's least significant difference (LSD) procedure. Results were deemed significant at $P < 0.05$.

3. Results

3.1 ECP toxicity testing and LD_{50} determination

The LD_{50} value was 3.3-µg protein.g^{-1} body weight (Table 1). Oysters injected with ECPs died rapidly, all recorded deaths occurring within 24-h post inoculation. No mortality was observed in the FSSW or uninjected control groups.

3.2 Hemocyte responses to different concentrations of ECPs

Phagocytic activity: After 1-h incubation, phagocytic activity was significantly affected by a concentration of ECPs as low as 2-µg.mL^{-1}, resulting in a 30% loss of hemocyte phagocytic activity (ANOVA, $P < 0.05$) (Fig. 3). Inhibition of phagocytosis by ECPs was dose-dependent and reached a maximum at a concentration of 16-µg.mL^{-1} (ANOVA, $P < 0.05$), with a decrease of 64.9% of the phagocytic ability, compared to the FSSW control. Concentrations of ECPs above 16-µg.mL^{-1} did not significantly result in an additional inhibition of phagocytic activity.

Adhesive capacity: After 3-h of incubation, hemocyte capacity for adhesion was significantly inhibited by ECPs at the lowest concentration (2-µg.mL^{-1}), with a decrease of 21% compared to the control (Kruskal-Wallis test, $P < 0.05$) (Fig. 4). This inhibition was observed to be similar for all the tested concentrations and was thus not dose-dependent.

ROS production: The synthesis of ROS increased with incubation time in control sub-samples for the two hemocyte sub-populations (i.e. granulocytes and hyalinocytes), this activity being higher in granulocytes compared to hyalinocytes after 60 and 120-min of incubation (Fig. 5). The ROS production of granulocytes significantly increased from a concentration of 64-µg.mL^{-1} of ECPs after 60 and 120-min of incubation, representing, respectively, an increase of 52.8% and 20.5% compared to the FSSW control (ANOVA, $P < 0.05$). Hyalinocytes were more sensitive than granulocytes, as the ROS production of hyalinocytes was significantly enhanced by 16-µg.mL^{-1} of ECPs (+ 25.5% compared to the control). After 120-min of incubation, a higher concentration of ECPs was necessary to obtain a significant increase in hyalinocyte ROS production. Indeed, DCF fluorescence significantly differed from the control at the same concentration of ECPs as that observed for granulocytes (i.e. 64-µg.mL^{-1}), with an increase of 30.5% (ANOVA, $P < 0.05$).

3.3 Minimal inhibitory concentration of ECPs

Phagocytic activity: as indicated in Fig. 6, this activity was significantly affected from a concentration of ECPs as low as 0.5-µg.mL^{-1}, with hemocytes retaining only 55.3% of their original phagocytic activity (ANOVA, $P < 0.05$). As previously observed, this inhibition appeared to be dose-dependent with a concentration of ECPs of 2-µg.mL^{-1} leading to a decrease of 75.8% of the phagocytic ability, compared to control hemocytes.

Adhesive capacity: as shown in Fig. 7, addition of ECPs to hemolymph induced a dose-dependent inhibition of hemocyte adhesive capacity over the range of 0.5 to 2-µg.mL^{-1}, the minimal inhibitory concentration of ECPs being 0.5-µg.mL^{-1} with a decrease of adhesive capacity of 5.1% compared to the control (ANOVA, $P < 0.05$).

3.4 Comparison of V. aestuarianus 01/32 live cells and ECPs on hemocyte responses

Phagocytic activity: confirming the results previously obtained, *V. aestuarianus* extracellular products significantly inhibited the phagocytic activity of *C. gigas* hemocytes in a dose-dependent manner (Fig. 8). For the same incubation time, live *V. aestuarianus* 01/32 cells at 50 bacteria per hemocyte did not induce any significant difference in phagocytic capability (Kruskal-Wallis test, $P < 0.05$).

Adhesive capacity: As in the previous experiment, ECPs inhibited hemocyte adhesion properties with doses of 2 and 32-µg.mL^{-1} after 3-h of incubation (Fig. 9). After exposure to live *V. aestuarianus* 01/32 cells, the percentage of adherent hemocytes did not significantly differ from that of the control (ANOVA, $P < 0.05$).

ROS production: As presented in Fig. 10, addition of ECPs at 32-µg.mL^{-1} led to a significant increase in granulocyte and hyalinocyte ROS production (+ 53.5% and + 50.4% after 60-min of incubation, + 57.4% and + 55.4% after 120-min of incubation, respectively) compared to the FSSW control. At 2-µg.mL^{-1} of ECPs, ROS production of granulocytes and hyalinocytes increased slightly, but not significantly. Conversely, stimulation or inhibition of hemocyte ROS production did not occur, in either sub-population, in response to *V. aestuarianus* 01/32 live cells, regardless of incubation time (ANOVA, $P < 0.05$).

4. Discussion

4.1 ECPs display lethality to C. gigas oysters

V. aestuarianus 01/32 ECP activities were evaluated *in vivo* by injection into the adductor muscle of *C. gigas* oysters. In this experimental system, the toxicity of *V. aestuarianus* 01/32 ECPs was clearly demonstrated. The calculated LD_{50} value (3.3-µg protein per g of body weight) was comparable to those reported for other marine mollusk pathogens of the *Vibrio* genus [36]. However, ECPs of *V. aestuarianus* 01/32 displayed a particularly strong toxic effect, as all recorded mortalities occurred within 24-h post inoculation. In comparison, the susceptibility of the small abalone *Haliotis diversicolor supertexta* to *V. parahaemolyticus* ECPs was weaker, since moribund animals were observed up to 7 days post challenge [37]. *V. aestuarianus* strain 01/32 secretes a highly-toxic material that could play an important role in virulence mechanisms.

4.2 ECPs inhibit hemocyte phagocytosis and adhesive capacities

Bivalve hemocytes spontaneously develop cytoplasmic extensions (pseudopods) and rapidly adhere when they are placed on flat surfaces. These abilities of hemocytes have been demonstrated to be affected by live cells of some *Vibrio* spp. known to be pathogenic to bivalves. For example, *V. alginolyticus* and *V. anguillarum* cells induced loss of pseudopods and cell rounding of *Mytilus edulis* hemocytes [38, 39]. Similarly, Choquet *et al*. [21] demonstrated, using a flow cytometry-based technique, that 3-h incubation of clam *R. philippinarum* hemocytes with *V. tapetis* live cells led to a marked reduction in hemocyte adhesive capabilities. This technique was adapted here to quantify the effects of *V. aestuarianus* 01/32 ECPs on the adhesive capabilities of oyster, *C. gigas,* hemocytes. Indeed, microscopic observations showed that ECPs produced by this *Vibrio* strain affected oyster hemocytes by causing cell rounding and lost of pseudopods (data not shown). The flow-cytometric assay, based on the counting of non-adherent hemocytes, allowed demonstration that a concentration of ECPs as low as 0.5-$\mu g.mL^{-1}$ resulted in a significant decrease in hemocyte adhesive capabilities. Previous assays with other *Vibrio* extracellular products have been performed in fish or human cell lines. For instance, *V. cholerae* Non-O1 was shown to produce a non-membrane damaging cytotoxin (NMDCY), also known as cell rounding factor, which caused rapid rounding of HeLa cultured cells [40]. ECPs of *V. tapetis* induced vacuolization of fish cells and several morphological alterations [41]. In bivalve mollusks, such observations of hemocyte alterations caused by *Vibrio* strains or their ECPs are scarce. To the best of our knowledge, this is the first report of a cytotoxic effect of ECPs on the adhesive capabilities of bivalve hemocytes.

To further examine effects of ECPs on *C. gigas* hemocytes, we investigated the ability of hemocytes treated *in vitro* with ECPs to engulf fluorescent latex beads by phagocytosis. Data obtained correlated well with adhesion results. After 1-h of exposure to ECPs, phagocytosis was significantly affected by a concentration of 0.5-µg.mL^{-1}, hemocytes maintaining only 55.3% of their original phagocytic activity. Furthermore, this inhibitory effect appeared to be dose-dependent for tested concentrations of ECPs. The process of engulfing foreign particles (i.e. phagocytosis) is driven by a finely-controlled rearrangement of the actin cytoskeleton, involving pseudopod formation, which is an essential part of this phagocytic activity [42]. Taken together, all results suggest a close relationship between the loss of cytoplasmic extensions, the reduction of adhesion properties, and the inhibition of phagocytosis attributable to ECPs. The results also indicate that *V. aestuarianus* strain 01/32 secretes one or more phagocytosis- and adhesion-inhibiting factors. Few examples of pathogenic *Vibrio* producing substances biologically active against marine bivalve hemocytes are known [38]. Furthermore, none of these bacterial substances were shown to possess anti-phagocytic activity. Such an interaction was only demonstrated in the lepidopteran model host *Manduca sexta*, for which previous research showed that the supernatant of the insect pathogenic bacterium *Photorhabdus* strain W14 was able to suppress the phagocytosis of bacteria by *Manduca* hemocytes [43, 44].

4.3 ECPs affect ROS production in C. gigas hemocytes

C. gigas hemocyte ROS production was activated in a dose-dependent manner, significantly from a concentration of ECPs of 64-µg.mL^{-1} for granulocytes whatever the incubation time, and for hyalinocytes from 16 µg.mL^{-1} and 64-µg.mL^{-1} after 60 and 120-min, respectively. These results are consistent with those previously obtained by Lambert *et al.* [45] showing that ECPs of *V. aestuarianus* strain 01/32 led to a significant stimulation of hemocyte ROS production. The observed increase in ROS production in both studies was higher than observed by Lambert *et al.* [25] after zymosan activation using the same measurement technique. These authors also proposed that ROS production is triggered after bleeding and hemolymph handling without use of conventional stimulants (phorbol-12-myristate 13-acetate (PMA) or zymosan). The ROS production by *C. gigas* hemocytes maintained in seawater after bleeding corresponds to our control treatment, but was not affected by ECPs at the range of concentrations affecting phagocytosis and adhesion.

The generation of cytotoxic ROS is a general protective mechanism in most, if not all, animal species. The production of these ROS compounds has been associated with phagocytosis in vertebrates as well as in bivalves [16, 46, 47]. As a consequence, an inhibition of hemocyte phagocytic processes could reasonably be expected to result in a subsequent

decrease in ROS production. Our data are, consequently, surprising and could appear contradictory, with clear inhibition of hemocyte adhesion and phagocytosis by ECPs. In many cases, bacteria evade hemocyte killing by preventing ROS production. For instance, different strains of *Vibrio*, known or suspected to be pathogenic to bivalves, were shown to inhibit the ROS production capacity of zymosan-stimulated hemocytes [25]. Considering the role of ECPs, Densmore *et al*. [48] demonstrated a dose-dependent relationship between the concentration of *R. salmoninarum* ECPs and the inhibition of ROS production by trout phagocytes, previously stimulated by addition of PMA. *In vitro* treatment of *Scophthalmus maximus* (L.) macrophages with *V. pelagius* ECPs caused a significant inhibition in the chemiluminescence (CL) response of PMA-activated phagocytes [49]. Therefore, the results obtained with ECPs from the bacterial strain 01/32 appear to suggest a different mechanism. Indeed, few studies report data on the ability of ECPs to exert cellular responses through the generation of ROS. Hofman *et al*. [50] and Falzano *et al*. [51] provided evidence that the cytotoxic necrotizing factor 1 (CNF-1), a protein toxin produced by pathogenic strains of *E. coli*, stimulated ROS production in human polymorphonuclear leukocytes (PMNL). This effect was shown to be associated with a decrease in PMNL phagocytic function [50, 51]. According to these authors, this excessive generation of ROS may enhance the virulence of *E. coli* infections by provoking damage to host epithelial cells [50].

Overall, data obtained here may support a model wherein ECPs impair hemocyte adhesive and phagocytic activities. Over-activation of ROS production may promote cytotoxicity in host hemocytes, allowing *V. aestuarianus* 01/32 cells to overcome cellular defenses of *C. gigas,* thereby facilitating bacterial growth and dissemination in host tissues. This excessive production of ROS, possibly toxic for host cells, should display the same toxicity for bacterial cells. However, most of bacteria possess antioxidants in the form of enzymes (e.g., superoxide dismutases, peroxydases). To counteract elevated levels of H_2O_2, it has been shown that many aerobic bacteria produce catalase, a high-molecular-weight protein whose primary function is to destroy H_2O_2, leaving H_2O and O_2 as by-products [52]. *Pseudomonas aeruginosa* was shown to possess high catalase activity, involved in resistance to increasing concentrations of H_2O_2 [52]. Bramble *et al.* [22] hypothesized that *L. anguillarum* catalase could suppressed the CL generated by *C. virginica* hemocytes. In a similar manner, one could assume that *V. aestuarianus* strain 01/32 possesses the ability to degrade the host-generated ROS by producing antioxidant enzymes. The production of such enzymes by the bacterium will be further investigated.

4.4 Viable V. aestuarianus 01/32 cells do not alter in vitro oyster immune parameters

As shown in the Results section, live bacteria demonstrated activity on neither hemocyte adhesive capacity and

phagocytosis, nor on ROS production when tested at a ratio of 50 bacteria per hemocyte and within a limited incubation time. According to this result, it could be hypothesized that the phagocytosis- and adhesion-inhibiting factor(s) could be mainly produced when the bacteria enter the exponential growth phase, i.e., are actively growing. This assumption is supported by the fact that bacterial cells were washed with FSSW before their addition to hemocytes, to eliminate all products secreted into the medium. Bacteria were subsequently exposed to hemocytes only for a short period of time (1-h for phagocytosis and ROS production assays, 3-h for adhesion assays). Moreover, ECPs used in this study were obtained by harvesting a 2 day-old culture, when bacteria reach the stationary phase of growth. As a consequence, it could be that the time of pre-incubation of bacteria with hemocytes may have been insufficient to allow the production and the release of these factors by live cells. Preliminary *in vivo* experiments (data not shown) have shown dramatic decreases in hemocyte adhesive and phagocytic properties of *V. aestuarianus* 01/32 infected oysters, compared to animals injected with FSSW. These observed effects occurred relatively slowly, requiring approximately 72-h post challenge to be significant. These data corroborate *in vitro* results presented in this study, and correlate well with the normal infection process, since this strain provokes an outbreak of mortality in *C. gigas* oysters 3 or 4 days after exposure (unpublished data). Assessing oyster immune parameters in response to a bacterial infection with *V. aestuarianus* 01/32 will be further investigated.

We describe in this work the toxicity to oysters of extracellular products from a pathogenic *Vibrio* strain and demonstrate the bacterium's capacity to reduce hemocyte adhesion properties and phagocytosis, and to increase the production of reactive oxygen species. These findings provide new insights into the cellular basis of bacteria-hemocyte interactions. This may help to explain how a bacterial pathogen succeeds in avoiding phagocytic engulfment to multiply to unsafe levels in oysters, ultimately leading to death.

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Figure captions

Fig. 1 Flow cytometer FL1 histogram plot of oyster hemocytes (Events) incubated with fluorescent latex beads. The line labeled M1 indicates the position of the marker used to assess the proportion of hemocytes containing 3 beads or more.

Fig. 2 Flow cytometer density plot of *C. gigas* hemocytes allowing the distinction of sub-populations (agranulocytes, granulocytes and hyalinocytes) according to their relative size (FSC) and complexity (SSC) after gating all active cells showing DCF fluorescence.

Fig. 3 Phagocytic activity of hemocytes (percentage of hemocytes containing 3 beads or more) submitted to increasing concentrations of ECPs ($\mu g.mL^{-1}$). Letters indicate significant difference between treatments (mean ± S.D.; N=4;

ANOVA $P < 0.05$).

Fig. 4 Percentage of adherent hemocytes submitted to increasing concentrations of ECPs ($\mu g.mL^{-1}$). Letters indicate significant difference between treatments (mean ± S.D.; N=4; Kruskal-Wallis, $P < 0.05$).

Fig. 5 Mean ROS production level (DCF fluorescence in arbitrary unit A.U.) of hemocytes submitted to increasing concentrations of ECPs ($\mu g.mL^{-1}$) at 60 and 120-min. Asterisk (*) indicates significant differences with FSSW control (mean ± S.D.; N=4; ANOVA, $P < 0.05$). (a) : granulocytes (b) : hyalinocytes

Fig. 6 Phagocytic activity of hemocytes (percentage of hemocytes containing three beads or more) submitted to low concentrations of ECPs (from 0.1 to 2-$\mu g.mL^{-1}$) . Letters indicate significant difference between treatments (mean ± S.D., N = 4, ANOVA $P<0.05$)

Fig. 7 Percentage of adherent hemocytes submitted to low concentrations of ECPs (from 0.1 to 2-$\mu g.mL^{-1}$) Letters indicate significant difference between treatments (mean ± S.D., N = 4, ANOVA $P < 0.05$)

Fig. 8 Phagocytic activity of hemocytes (percentage of hemocytes containing three beads or more) after 1-h exposure to ECPs (2 and 32-$\mu g.mL^{-1}$) or to *V. aestuarianus* 01/32 live cells (50 bacteria/ hemocyte). Letters indicate significant difference between treatments (mean ± S.D.; N=4; Kruskal-Wallis, $P < 0.05$).

Fig. 9 Percentage of adherent hemocytes after 3-h exposure to ECPs (2 and 32-$\mu g.mL^{-1}$) or to *V. aestuarianus* 01/32 live cells (50 bacteria/ hemocyte). Letters indicate statistical difference between treatments (mean ± S.D.; N=4; ANOVA, $P < 0.05$).

Fig. 10 Mean ROS production level (DCF fluorescence in arbitrary unit) of hemocytes stimulated by ECPs (2 and 32-$\mu g.mL^{-1}$) or *V. aestuarianus* 01/32 live cells (50 bacteria/ hemocyte). Letters indicate statistical difference with FSSW condition (control) (mean ± S.D.; N=4; ANOVA, $P < 0.05$). (a) : granulocytes (b) : hyalinocytes

Tables

Table 1: Virulence tests of ECPs produced by *V. aestuarianus* 01/32 strain injected in a volume of 0.2-mL into *C. gigas* oysters weighing 5 ± 1 g

Dose per oyster ($\mu g.g^{-1}$ body weight)	No of dead oysters [a]	Relative virulence [b]
10	20	100 %
5	14	78.6 %
2.5	8	30.8 %
1.25	0	0 %
LD_{50}	3.3 $\mu g. g^{-1}$ body weight	

a : expressed as the number of oysters dying over the total number of oysters in the treatment. Overall results of duplicate trial are presented.

b : expressed by dividing the cumulative number of dead oysters with the cumulative total number of oysters injected.

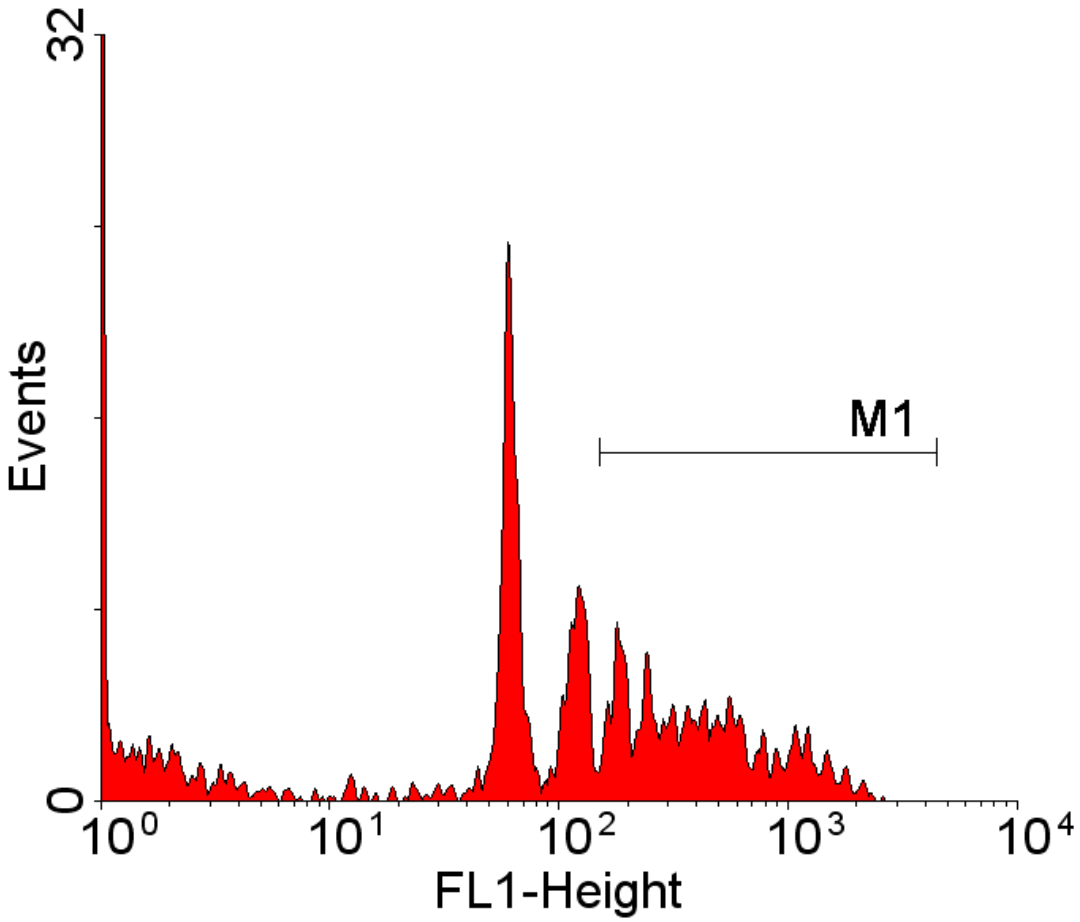


Fig. 1

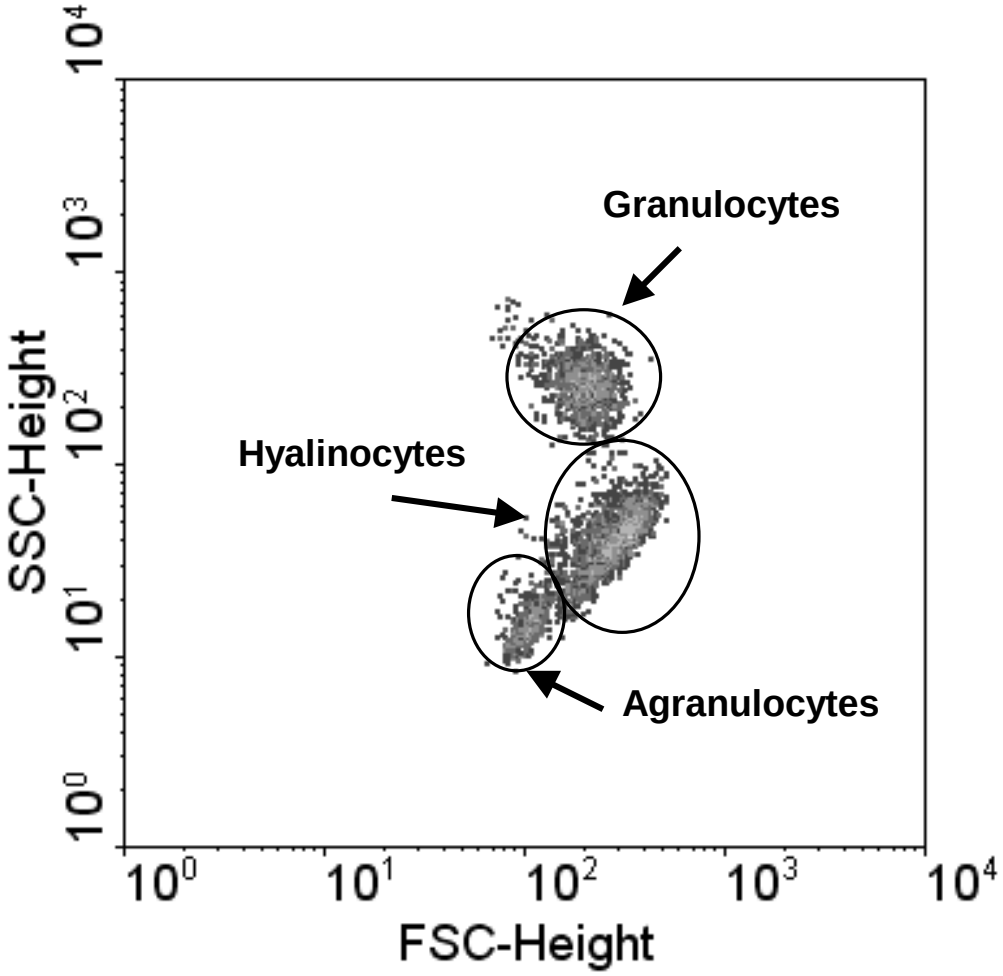

Granulocytes
Hyalinocytes
Agranulocytes
SSC-Height
FSC-Height
10^0
10^1
10^2
10^3
10^4

Fig. 2

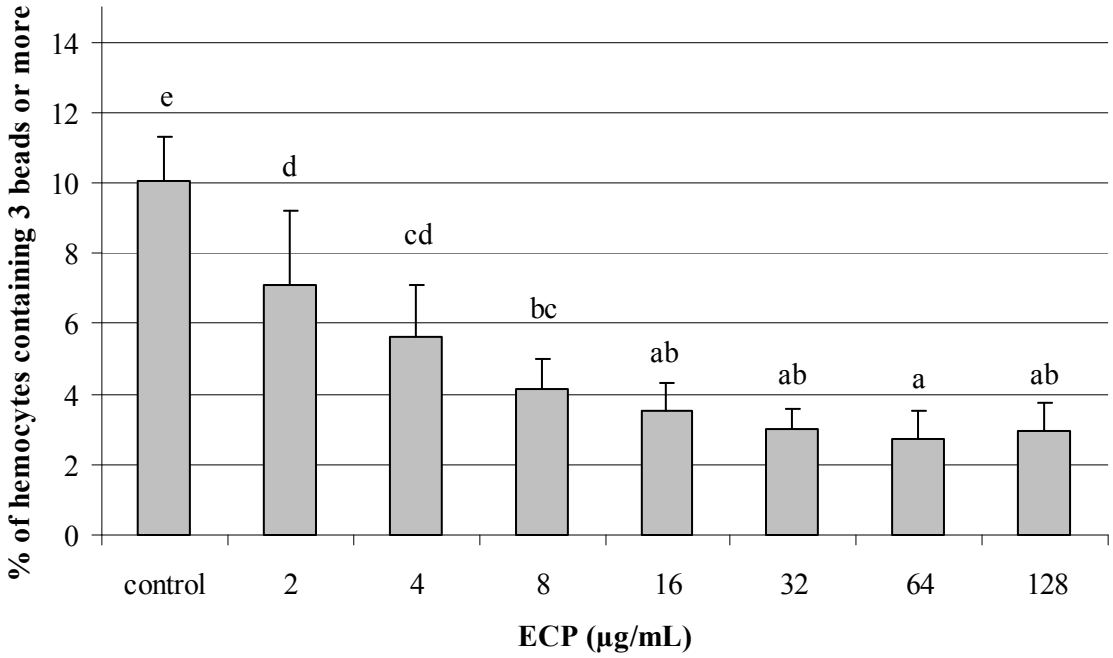

% of hemocytes containing 3 beads or more
14
12
10
8
6
4
2
0
e
d
cd
bc
ab
ab
a
ab
control
2
4
8
16
32
64
128
ECP (µg/mL)

Fig. 3

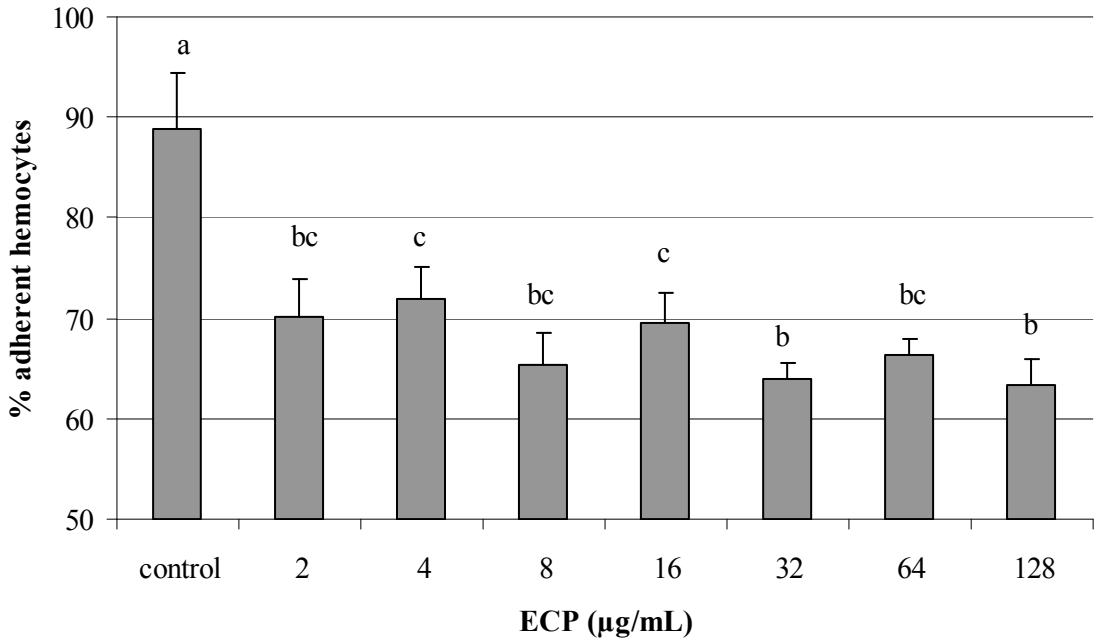
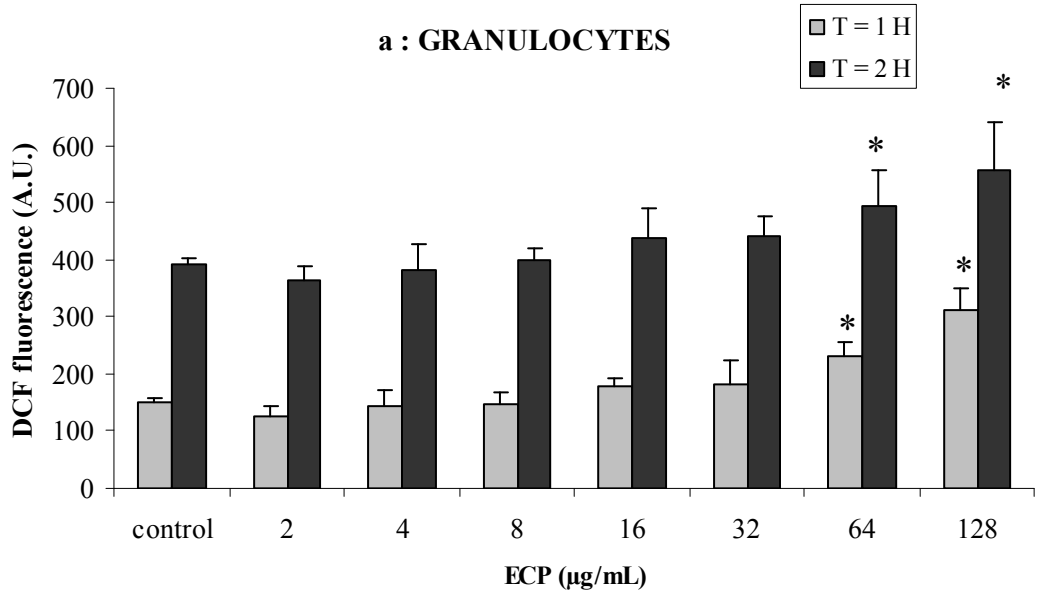

100
90
80
70
60
50
% adherent hemocytes
a
bc
c
bc
c
b
bc
b
control
2
4
8
16
32
64
128
ECP (µg/mL)

Fig. 4


a : GRANULOCYTES
T = 1 H
T = 2 H
DCF fluorescence (A.U.)
0
100
200
300
400
500
600
700
control
2
4
8
16
32
64
128
ECP (µg/mL)

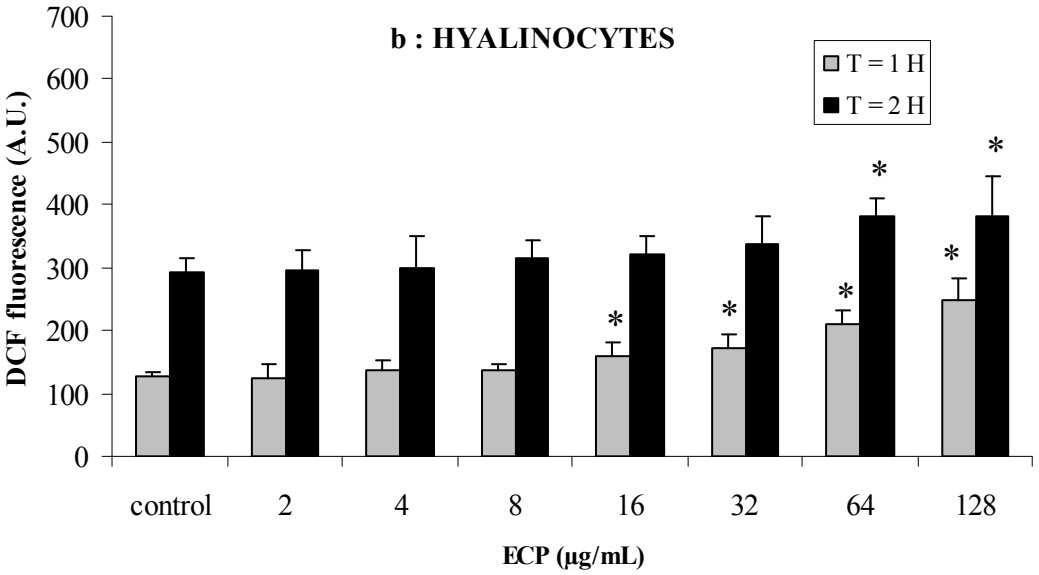

b : HYALINOCYTES
T = 1 H
T = 2 H
DCF fluorescence (A.U.)
0
100
200
300
400
500
600
700
control
2
4
8
16
32
64
128
ECP (µg/mL)

Fig. 5

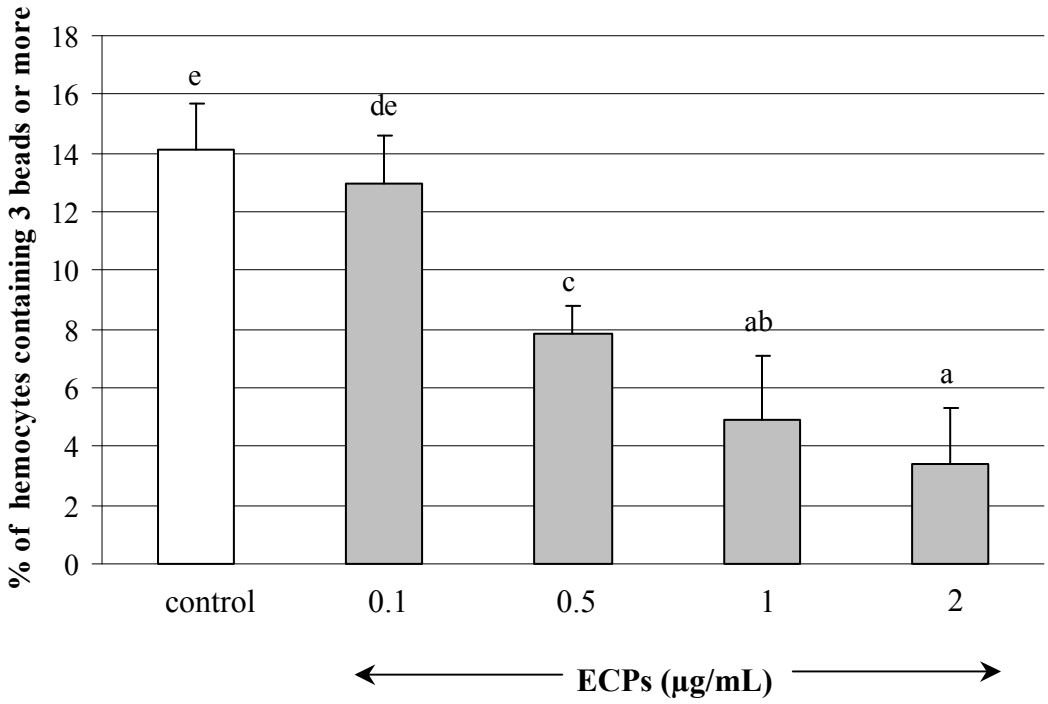

% of hemocytes containing 3 beads or more
18
16
14
12
10
8
6
4
2
0
e
de
c
ab
a
control
0.1
0.5
1
2
ECPs (µg/mL)

Fig. 6

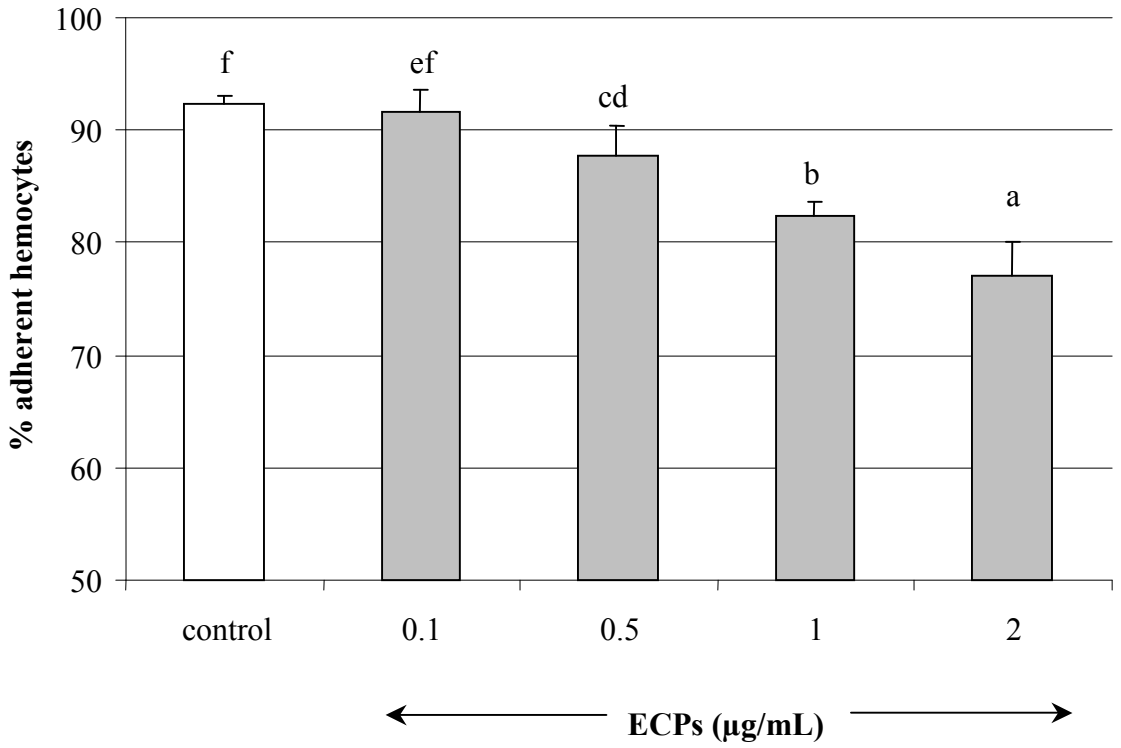

100
90
80
70
60
50
% adherent hemocytes
f
ef
cd
b
a
control
0.1
0.5
1
2
ECPs (µg/mL)

Fig. 7

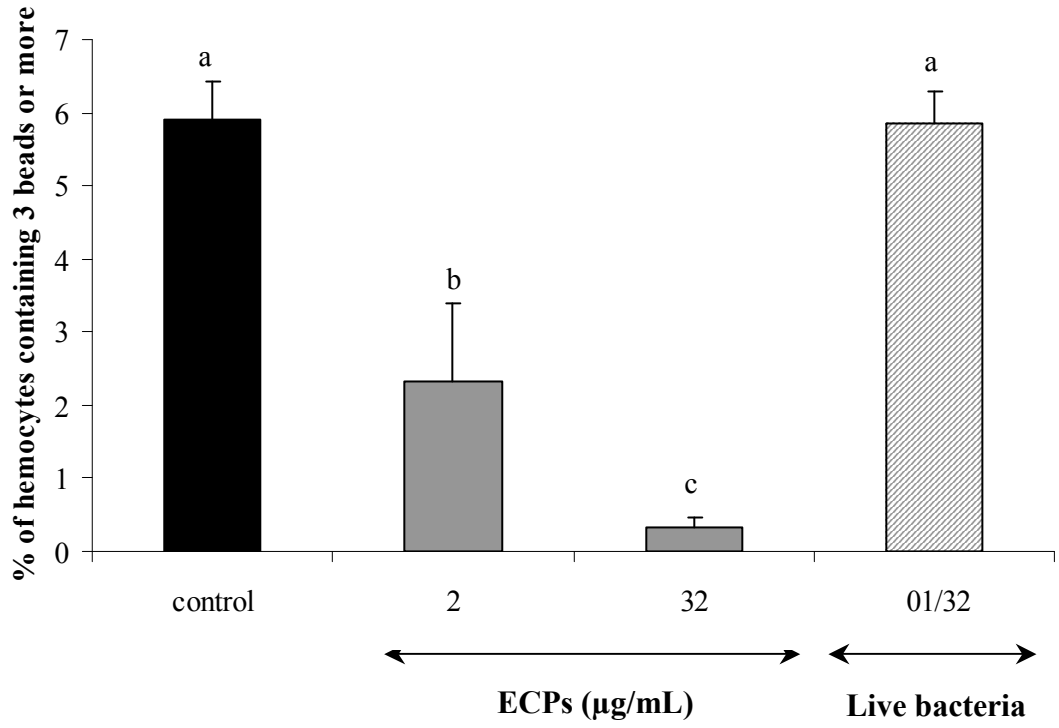

% of hemocytes containing 3 beads or more
0
1
2
3
4
5
6
7
a
b
c
a
control
2
32
01/32
ECPs (µg/mL)
Live bacteria

Fig. 8

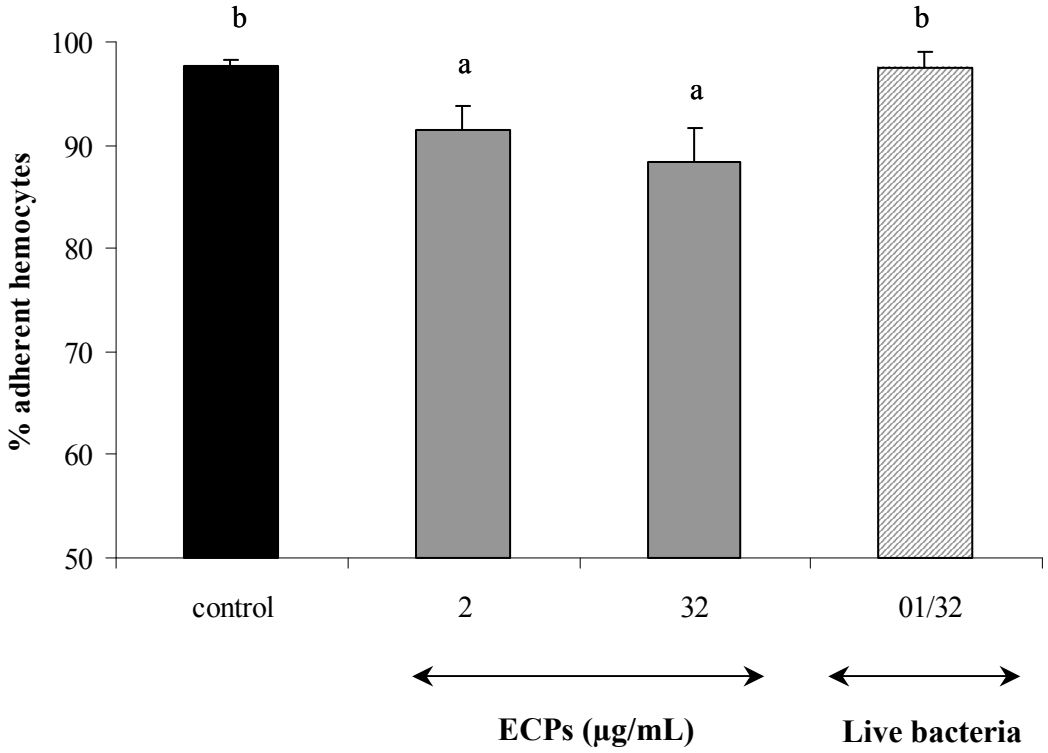

b
a
a
b
100
90
80
70
60
50
% adherent hemocytes
control
2
32
01/32
ECPs (µg/mL)
Live bacteria

Fig. 9

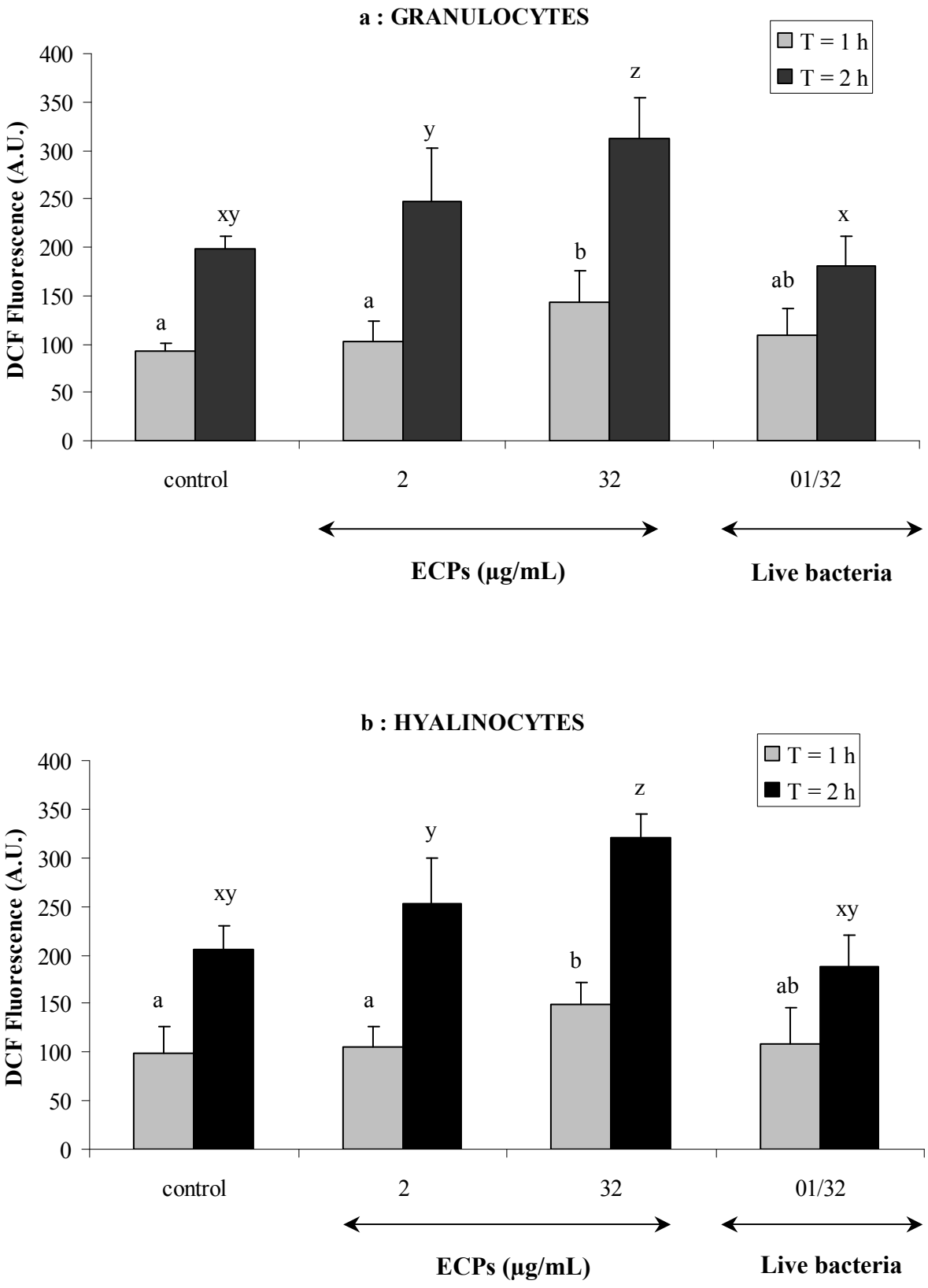

a : GRANULOCYTES
T = 1 h
T = 2 h
DCF Fluorescence (A.U.)
400
350
300
250
200
150
100
50
0
a
xy
a
y
b
z
ab
x
control
2
32
01/32
ECPs (µg/mL)
Live bacteria
b : HYALINOCYTES
T = 1 h
T = 2 h
DCF Fluorescence (A.U.)
400
350
300
250
200
150
100
50
0
a
xy
a
y
b
z
ab
xy
control
2
32
01/32
ECPs (µg/mL)
Live bacteria

Fig. 10