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# Responses of *Mytilus galloprovincialis* to challenge with the emerging marine pathogen *Vibrio coralliilyticus*

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ABSTRACT

*Vibrio coralliilyticus* has emerged as a coral pathogen of concern throughout the Indo-Pacific reef. The interest towards understanding its ecology and pathogenic potential has increased since *V. coralliilyticus* was shown to be strongly virulent also for other species; in particular, it represents a serious threat for bivalve aquaculture, being one of the most important emerging pathogen responsible for oyster larval mortalities worldwide. *V. coralliilyticus* has a tightly regulated temperature-dependent virulence and it has been related to mass mortalities events of benthic invertebrates also in the temperate northwestern Mediterranean Sea. However, no data are available on the effects of *V. coralliilyticus* in the mussel *Mytilus galloprovincialis*, the most abundant aquacultured species in this area. In this work, responses of *M. galloprovincialis* to challenge with *V. coralliilyticus* (ATCC BAA-450) were investigated. In vitro, short term responses of mussel hemocytes were evaluated in terms of lysosomal membrane stability, bactericidal activity, lysozyme release, ROS and NO production, and ultrastructural changes, evaluated by TEM. In vivo, hemolymph parameters were measured in mussels challenged with *V. coralliilyticus* at 24h p.i. Moreover, the effects of *V. coralliilyticus* on mussel early embryo development (at 48 hpf) were evaluated. The results show that both in vitro and in vivo, mussels were unable to activate immune response towards *V. coralliilyticus*, and that challenge mainly induced lysosomal stress in the hemocytes. Moreover, *V. coralliilyticus* showed a strong and concentration-dependent embryotoxicity. Overall, the results indicate that, although *M. galloprovincialis* is considered a resistant species to vibrio infections, the emerging pathogen *V. coralliilyticus* can represent a potential threat to mussel aquaculture.

## 1. Introduction

Marine bivalves, due to their filter-feeding habit, accumulate large numbers of bacteria from the harvesting waters. Bivalves possess both cellular and humoral defence mechanisms that co-operate to kill and eliminate infecting bacteria [1,2]. However, some bacteria can be pathogenic to the bivalve host, in particular those belonging to the genus *Vibrio*. Pathogenic vibrios can mainly affect larval stages of cultured bivalves, and are also involved in diseases of juveniles and adults [3–5]. The *Vibrio* species with importance for bivalve hatcheries due to the known pathogenicity for larvae and spat have been recently summarized [6]. These include species from the *Anguillarum*, *Coralliilyticus*, *Harveyi*, *Orientalis*, *Pectenicida* and *Splendidus* clades.

*Vibrio coralliilyticus* has emerged as a coral pathogen of concern throughout the Indo-Pacific reef [7,8]. The interest towards understanding its ecology and pathogenic potential has increased since *V. coralliilyticus* was shown to be strongly virulent also for other species, such as unicellular algae [9,10], flies [10,11], rainbow trout (*Oncorhynchus mykiss*) and larval brine shrimp (*Artemia* spp.) [12]. Moreover, *V. coralliilyticus* represents a serious threat for bivalve aquaculture, being one of the most important emerging pathogen responsible for oyster larval mortalities worldwide [13–15]. *V. coralliilyticus* has been also associated with outbreaks of vibriosis in several other bivalve species, such as hard clam (*Mercenaria mercenaria*), New Zealand green-lipped mussel (*Perna canaliculus*), Atlantic bay scallop (*Argopecten irradians*) and naval shipworm (*Teredo navalis*) [13,15]. In the purple gorgonian *Paramuricea clavata* of the temperate north-western Mediterranean Sea, experimental infections with *V. coralliilyticus* showed a tightly regulated temperature-dependent virulence, in a range of temperatures consistent with those observed during the occurrence of mortality episodes in the field [16]. However, no information is available of the effects of *V. coralliilyticus* in the Mediterranean mussel

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*Mytilus galloprovincialis*, which represents the most abundant aquacultured species in this area.

Although *Mytilus* spp., including *M. galloprovincialis*, is particularly resistant to bacterial infections, it shows a remarkable specificity of the immune response towards different *Vibrio* spp. and strains, as demonstrated both *in vitro* and *in vivo* studies in adults [2,17,18]. In contrast, little information is available on the possible vibrio pathogens affecting *Mytilus* embryo development [19].

In this work, data are presented on immune responses of *M. galloprovincialis* to challenge with the emerging marine pathogen *V. coralliilyticus*. *In vitro*, short term responses of mussel hemocytes to *V. coralliilyticus* were evaluated in terms of lysosomal membrane stability (LMS), bactericidal activity, extracellular lysozyme release, Reactive Oxygen Species (ROS) and Nitric oxide (NO) production. The effects on hemocyte morphology were also investigated by Transmission Electron Microscopy (TEM). *In vivo*, hemocyte LMS, ROS production and serum lysozyme activity were measured in mussels challenged with *V. coralliilyticus* at 24 h post-injection. Moreover, the effects of *V. coralliilyticus* on mussel early embryo development (at 48 h post fertilization-hpf) were evaluated.

## 2. Methods

### 2.1. Mussels and bacteria

Mussels (*Mytilus galloprovincialis* Lamarck, 1819), 4–5 cm long, were purchased from an aquaculture farm (Arborea-OR, Italy) in October 2017 and kept for 1 day in static tanks containing aerated artificial sea water (ASW), salinity 36 ppt (1 L/mussel) at 18 °C. Hemolymph was extracted from the posterior adductor muscle using a sterile 1 mL syringe with an 18 G1/2″ needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 mL Falcon tubes at 18 °C. Hemolymph serum was obtained by centrifugation of whole hemolymph at 100 × *g* for 10 min, and the supernatant was sterilized through a 0.22 μm-pore filter.

*V. coralliilyticus* ATCC BAA-450 and *V. coralliilyticus* TAV24 (isolated from diseased *Paramuricea clavata* colonies [16]) were cultured in Zobell Marine Broth 2216 (Difco Laboratories) at 20 °C under static conditions; after overnight growth, cells were harvested by centrifugation (4500 × *g*, 10 min), washed three times with artificial seawater (ASW), and resuspended to obtain a concentration of about $10^8$ CFU/mL (determined spectrophotometrically as an $Abs_{600} = 1$). Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar (Conda Lab, Spain) was used throughout the experiments.

### 2.2. In vitro challenge of Mytilus hemocytes with V. coralliilyticus

Hemocyte monolayers were prepared as previously described [18,20] and incubated at 18 °C with suspensions of *V. coralliilyticus* suitably diluted in hemolymph serum at different concentrations ($5 \times 10^5$, $5 \times 10^6$, $5 \times 10^7$ CFU/mL), for different periods of times, depending on the endpoint measured. Untreated hemocyte samples in serum were run in parallel.

#### 2.2.1. Determination of lysosomal membrane stability

Lysosomal membrane stability-LMS in hemocyte monolayers was evaluated by the Neutral Red Retention Time (NRRT) assay as previously described [17,18,20]. Hemocyte monolayers on glass slides were pre-incubated for 30 min with different concentrations of *V. coralliilyticus*. Hemocyte monolayers were washed out and incubated with 20 μL of a neutral red (NR) (Sigma-Aldrich, Milan, Italy) solution (final concentration 40 μg/mL from a stock solution of NR 20 mg/mL DMSO-dimethylsulfoxide). After 15 min, excess of dye was washed out, 20 μL of ASW was added, and slides were sealed with a coverslip. Every 15 min, slides were examined under optical microscope and the percentage of cells showing loss of dye from lysosomes in each field was evaluated. For each time point, 10 fields were randomly observed, each containing 8–10 cells. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking, i.e. the cytosol becoming red and the cells rounded. All incubations were carried out at 18 °C.

For comparison, LMS was evaluated using the Mediterranean strain *V. coralliilyticus* TAV24 [16] in the same experimental conditions as described above.

#### 2.2.2. Bactericidal activity

Bactericidal activity was evaluated as previously described [20,21]. Hemocyte monolayers were incubated with different concentrations of *V. coralliilyticus* at 18 °C, for different periods of time. Immediately after the inoculum (T = 0) and after 60 and 90 min of incubation, supernatants were collected and hemocytes were lysed by adding filter sterilized ASW supplemented with 0.05% Triton x-100 and by 10 s agitation. Supernatants and lysates were pooled and tenfold serial diluted in ASW. Aliquots (100 μL) of the diluted samples were plated onto TCBS Agar. After overnight incubation at 20 °C, the number of colony-forming units (CFU) per hemocyte monolayer (representing live, culturable bacteria) was determined. Percentages of killing were determined in comparison to values obtained at zero time. The number of CFU in control hemocytes never exceeded 0.1% of those enumerated in experimental samples.

#### 2.2.3. Lysozyme release, ROS and NO production

For these endpoints, hemocytes were incubated with suspensions of *V. coralliilyticus* in serum at $5 \times 10^6$ CFU/mL. Lysosomal enzyme release by mussel hemocytes was evaluated by measuring lysozyme activity in the extracellular medium as previously described [20]. Lysozyme activity in aliquots of serum of control hemocytes and hemocytes incubated *V. coralliilyticus* for different periods of time (from 5 to 30 min), was determined spectrophotometrically at 450 nm using a suspension of *Micrococcus lysodeiktícus* (15 mg/100 mL in 66 mM phosphate buffer, pH 6.4). Hen eggwhite (HEW) lysozyme was used as a concentration reference and lysozyme activity was expressed as HEW lysozyme equivalents (U mg $protein^{-1}$ $mL^{-1}$). Protein content was determined according to the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard. Data are expressed as percentage of control values.

Extracellular generation of reactive oxygen species (ROS) was measured by the reduction of cytochrome c as previously described [18]. Aliquots of hemocyte suspension were incubated for 30 min with cytochrome c solution (75 mM ferricytochrome c in TBS), with or without *V. coralliilyticus*. Cytochrome c in TBS was used as a blank. Samples were read at 550 nm and the results expressed as changes in OD per mg protein.

Nitric oxide (NO) production was evaluated as described previously [18] by the Griess reaction, which quantifies the nitrite ($NO_2^-$) content of supernatants. Aliquots of hemocyte suspensions were incubated at 18 °C with or without bacterial suspension of *V. coralliilyticus* for 2 h. After the incubation, samples were frozen and stored at −80 °C until analysis. Before analysis, samples were thawed and centrifuged (12000 × *g* for 30 min at 4 °C). Aliquots of supernatants were incubated for 10 min in the dark with 1% (w/v) sulphanilamide in 5% $H_3PO_4$ and 0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride. Samples were read at 540 nm, and the molar concentration of $NO_2^-$ in the sample was calculated from standard curves generated using known concentrations of sodium nitrite. Data are expressed as nitrite accumulation per protein content, determined according to the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard.

#### 2.2.4. Transmission electron microscopy

TEM of mussel hemocytes was carried out as previously described [20]. Hemocyte monolayers were seeded on glass chamber slides for

20 min at 18 °C (Lab-Tek, Nunc, 177380), and incubated with *V. coralliilyticus* ($5 \times 10^6$ CFU/mL in hemolymph serum) for 5, 15 and 30 min. Samples were washed out with 0.1 M cacodylate buffer in ASW and fixed in 0.1 M cacodylate buffer in ASW containing 2.5% glutaraldehyde in ASW, for 1 h at room temperature. The cells were postfixed in 1% osmium tetroxide in ASW for 10 min and 1% uranyl acetate in ASW for 1 h. Subsequently, samples were dehydrated through a graded ethanol series and embedded in epoxy resin (Poly-Bed; Polysciences, Inc., Warrington, PA) overnight at 60 °C. About 50 cells per sample were observed by F20 Tecnai electron microscope (Philips, Eindhoven, The Netherlands), and representative images were taken with an Eagle CCD camera and iTEM software and processed with Adobe Photoshop CS2.3.2.

### 2.3. In vivo challenge of adult mussels with V. coralliilyticus

Mussels were kept for 24 h in static tanks containing aerated artificial sea water (1 L/mussel) at 18 °C. Mussels were *in vivo* challenged by injection of live *V. coralliilyticus* into the posterior adductor muscle, as previously described [20], with 50 μL of a bacterial suspension containing $1 \times 10^8$ CFU/mL in phosphate buffered solution isotonic to hemolymph (PBS−NaCl: 2 mM $KH_2HPO_4$, 10 mM $Na_2HPO_4$, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4), in order to obtain a nominal concentration of $5 \times 10^6$ CFU/mussel). Control mussels were injected with PBS−NaCl. After challenge, mussels were returned to sea water. At 24 h post injection (p.i.), hemolymph was collected from the posterior adductor muscle of 4 pools of 4 mussels each. No mortality was observed during the experiments.

At 24 h p.i. in hemolymph samples from control and vibrio-injected mussels, hemocyte LMS and ROS production, soluble lysozyme activity, as well as bacterial counts, evaluated as number of CFU/mL of whole hemolymph, were determined as described above.

### 2.4. Effects of V. coralliilyticus on embryo development

Sexually mature mussels (*M. galloprovincialis* Lamarck, 1819), purchased from an aquaculture farm in the Ligurian Sea (La Spezia, Italy) between November and March, were transferred to the laboratory and acclimatized in static tanks containing aerated artificial sea water [22], pH 7.9–8.1, 36 ppt salinity (1 L/animal), at 18 ± 1 °C. Mussels were utilized within 2 days for gamete collection. Mussels were kept in tanks until they began to spontaneously spawn. Then each individual was immediately placed in single 250 mL beakers containing 200 mL of aerated ASW until complete gamete emission. After spawning, mussels were removed from beakers and sperms and eggs were sieved through 50 μm and 100 μm meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted microscope. For each experiment, eggs and sperm from two individuals were selected and counted to give a single pairing. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 96-microwell plates (Costar, Corning Incorporate, NY, USA). After 30 min fertilization success (n. fertilized eggs/n. total eggs x 100) was verified by microscopical observation (> 85%).

The 48-h embryotoxicity assay [22] was carried out in 96-microwell plates as described by Fabbri et al. [23]. Aliquots of 20 μL of suspensions of *V. coralliilyticus* (obtained from a $10^7$ CFU/mL stock suspension), suitably diluted in ASW, were added to fertilized eggs in each microwell to reach the nominal final concentrations ($10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ CFU/mL) in a 200 μL volume. At each dilution step, all suspensions were immediately vortexed prior to use. Microplates were gently stirred for 1 min, and then incubated at 18 ± 1 °C for 48 h, with a 16 h:8 h light:dark photoperiod. All the following procedures were carried out following ASTM [22]. At the end of the incubation time, samples were fixed with buffered formalin (4%). All larvae in each well were examined by optical and/or phase contrast microscopy using an inverted Olympus IX53 microscope (Olympus, Milano, Italy) at 40X, equipped with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). Observations were carried out by an operator blind to the experimental conditions. A larva was considered normal when the shell was D-shaped (straight hinge) and the mantle did not protrude out of the shell, and malformed if had not reached the stage typical for 48 hpf (trocophore or earlier stages) or when some developmental defects were observed (concave, malformed or damaged shell, protruding mantle). The acceptability of test results was based on controls for a percentage of normal D-shell stage larvae > 75% [22]. Moreover, in each sample the percentage of malformed D-veligers, immature veligers, and trocophorae was evaluated.

### 2.5. Data analysis

Data from hemocyte and hemolymph parameters are the mean ± SD of at least 4 independent experiments with each assay performed in triplicate. Statistical analyses were performed by Mann-Whitney *U* test using the GraphPad Instat software.

Embriotoxicity test data, representing the mean ± SD of 4 independent experiments, carried out in 6 replicate samples in 96-microwell plates, were analyzed by ANOVA plus Tukey's post test. The $EC_{50}$ was defined as the concentration causing 50% reduction in the embryogenesis success, and their 95% confidence intervals (CI) were calculated by PRISM 5 software (GraphPad Prism 5 software package, GraphPad Inc.).

## 3. Results

### 3.1. Effects of in vitro challenge with V. coralliilyticus on hemocyte functional parameters

As shown in Fig. 1A, incubation with *V. coralliilyticus* ATCC BAA-450 for 30 min induced a dose-dependent decrease in hemocyte LMS, evaluated by the NRRT assay, with respect to controls. The lowest concentration tested ($5 \times 10^5$ CFU/mL) was ineffective, while a moderate decrease was observed at $5 \times 10^6$ CFU/mL (−25%; $p < 0.05$). At the highest concentration ($5 \times 10^7$ CFU/mL) lysosomal membranes were completely destabilized (−98%; $p < 0.01$). Interestingly, similar results were obtained with the Mediterranean strain *V. coralliilyticus* TAV24 (Fig. 1A).

The capacity of mussel hemocytes to kill *V. coralliilyticus* ATCC BAA-450 was investigated using a bactericidal assay that evaluates the number of live, culturable bacteria at different times of incubation (Fig. 1B). Hemocytes were incubated with *V. coralliilyticus*, at the same concentrations utilized in the LMS assay, and the results are reported as % of killed bacteria with respect to the inoculum. The results clearly show a dose-dependent bactericidal activity towards *V. coralliilyticus*. At $5 \times 10^5$ CFU/mL, *V. coralliilyticus* was efficiently killed by mussel hemocytes (from 40% at 60 min to 65% at 90 min; $p < 0.05$). A lower percentage of killing was observed at the concentration of $5 \times 10^6$ CFU/mL (20% at both 60 and 90 min; $p < 0.05$). At the highest concentration tested ($5 \times 10^7$ CFU/mL) no significant bactericidal activity was recorded (less than 10% at 90 min).

On the basis of these results, subsequent experiments to evaluate other immune parameters were carried out using a concentration of bacteria of $5 \times 10^6$ CFU/mL, and the results are reported in Fig. 2. Immediately after addition of ATCC BAA-450 bacteria, a significant increase in extracellular lysozyme activity was observed with respect to controls (+37%, $p < 0.05$). No differences were measured at subsequent times of incubation. *V. coralliilyticus* ATCC BAA-450 did not affect extracellular ROS production (B) or nitrite accumulation (C) after 30 min and 2 h, respectively.

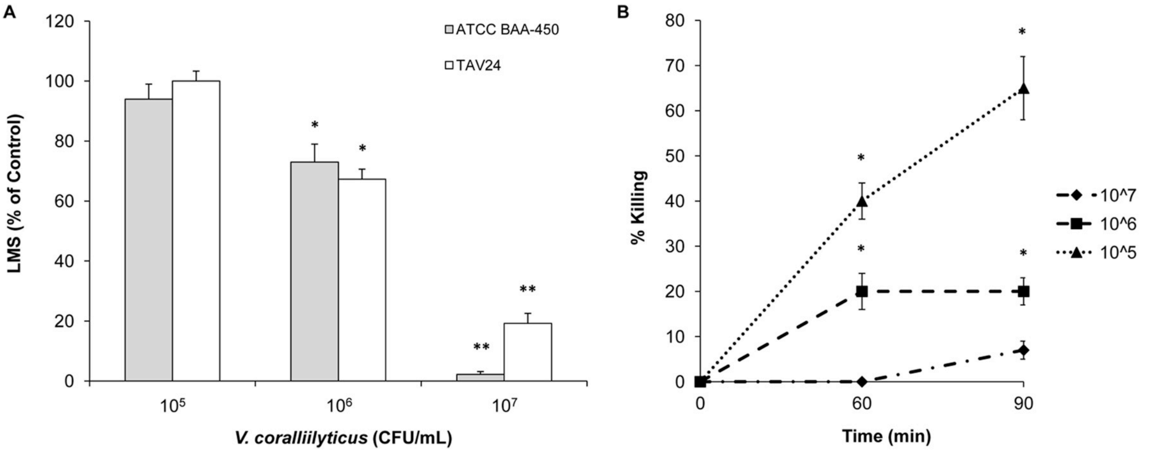

**Fig. 1. *In vitro* effects of *V. coralliilyticus* on lysosomal membrane stability-LMS and bactericidal activity.**
A) Hemocyte monolayers were treated with different concentrations ($5 \times 10^5$, $5 \times 10^6$, $5 \times 10^7$ CFU/mL) of *V. coralliilyticus* ATCC BAA-450 or *V. coralliilyticus* TAV24 for 30 min and LMS was evaluated as described in Methods. Data, expressed as percent values with respect to controls and representing the mean ± SD of 4 experiments in triplicate, were analyzed by Mann-Whitney *U* test ($* = p < 0.05$; $** = p < 0.01$). B) Hemocytes were incubated for different periods of time (60–90 min) with *V. coralliilyticus* ATCC BAA-450, at the same concentrations utilized in the LMS assay, and the number of viable, cultivable bacteria (CFU) per monolayer was evaluated. Percentages of killing were determined in comparison to values obtained at zero time. Data are the mean ± SD of at least 4 experiments performed in triplicate and statistical analyses were perfomed by Mann-Whitney *U* test ($* = p < 0.05$).

### 3.2. *Effects of in vitro challenge with V. coralliilyticus on hemocyte ultrastructure*

The effects of challenge with *V. coralliilyticus* ATCC BAA-450 ($5 \times 10^6$ CFU/mL) on the morphology of mussel hemocytes were observed by TEM at different times of incubation (5, 15 and 30 min) and representative images are reported in Fig. 3. Fig. 3A shows *V. coralliilyticus* ATCC BAA-450 before the addition to the hemocytes. A control hemocyte is shown in Fig. 3B; as previously reported [18,20], in hemocyte monolayers control cells are mainly represented by granulocytes, whose cytoplasm is filled by small intracellular granules of different electron densities.

*V. coralliilyticus* induced morphological changes in the hemocytes at the plasma membrane and cytoplasmic level as soon as 5 min from addition. Some cells formed irregular pseudopodial extensions (Fig. 3C) (P), while others showed a more flattened shape, with the cell membrane lining portions of empty cytoplasm (Fig. 3D) (arrowhead). Different ultrastructural changes were more evident at 15 min post-infection. In addition to the formation of long pseudopodia, *V. coralliilyticus* mainly affected the intracellular vacuolar system, as shown by the appearance of enlarged vacuoles of heterogeneous content (HV), empty vacuoles (EV), or vacuoles containing granular material (GV) (Fig. 3E and F). At 30 min (Fig. 3G and H), large electron dense vacuoles of heterogeneous content (HV) were observed, suggesting lysosomal fusion events, together with empty vacuoles and irregular plasma membrane surfaces (arrowheads).

### 3.3. *Effects of in vivo challenge with V. coralliilyticus on hemolymph parameters*

Mussels were injected with *V. coralliilyticus* ATCC BAA-450 in order to reach a nominal concentration of $5 \times 10^6$ CFU/mL of hemolymph. Samples were collected after 24 h p.i. and hemocyte LMS, serum lysozyme activity and ROS production were evaluated, as well as bacterial cell counts in whole hemolymph samples. The results show that *in vivo* challenge with *V. coralliilyticus* lead to a moderate but significant decrease in LMS at 24 h p.i. (−23%; $p < 0.05$) (Fig. 4A), comparable to that observed in *in vitro* experiments. No increases in serum lysozyme activity (Fig. 4B) and hemocyte ROS production (Fig. 4C) were observed; interestingly, the basal levels of ROS were even reduced with respect to controls (−24%, $p < 0.05$). Finally, in *V. coralliilyticus*-injected mussels, *Vibrio* counts were significantly higher (about 7-folds; $p < 0.01$) in hemolymph collected at 24 h p.i., compared to those in hemolymph collected immediately after infection (T = 0) (Fig. 4D).

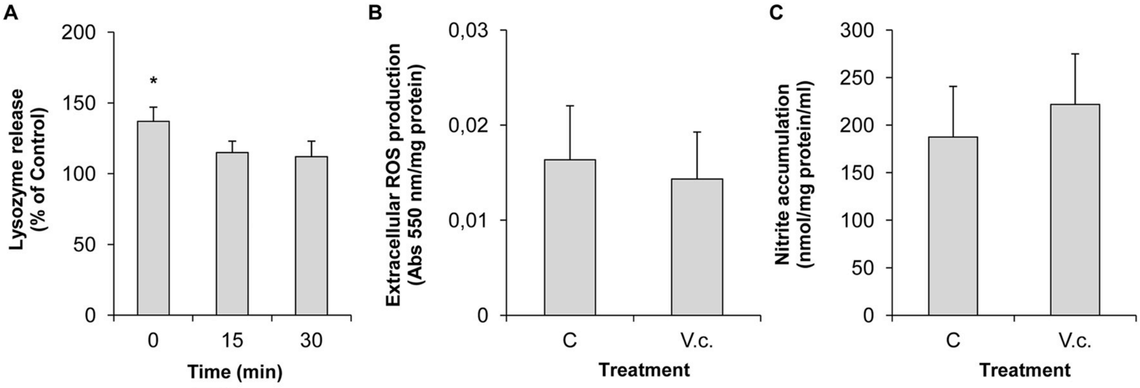

**Fig. 2. *In vitro* effects of *V. coralliilyticus* on functional parameters of *Mytilus* hemocytes. Lysosomal enzyme release.**
(A), extracellular ROS production (B) and NO accumulation (C) were evaluated after incubation with *V. coralliilyticus* (*V.c.*) at $5 \times 10^6$ CFU/mL in hemolymph serum. Data are the mean ± SD of at least 4 experiments performed in triplicate. Statistical analyses were performed by Mann-Whitney *U* test ($* = p < 0.05$).

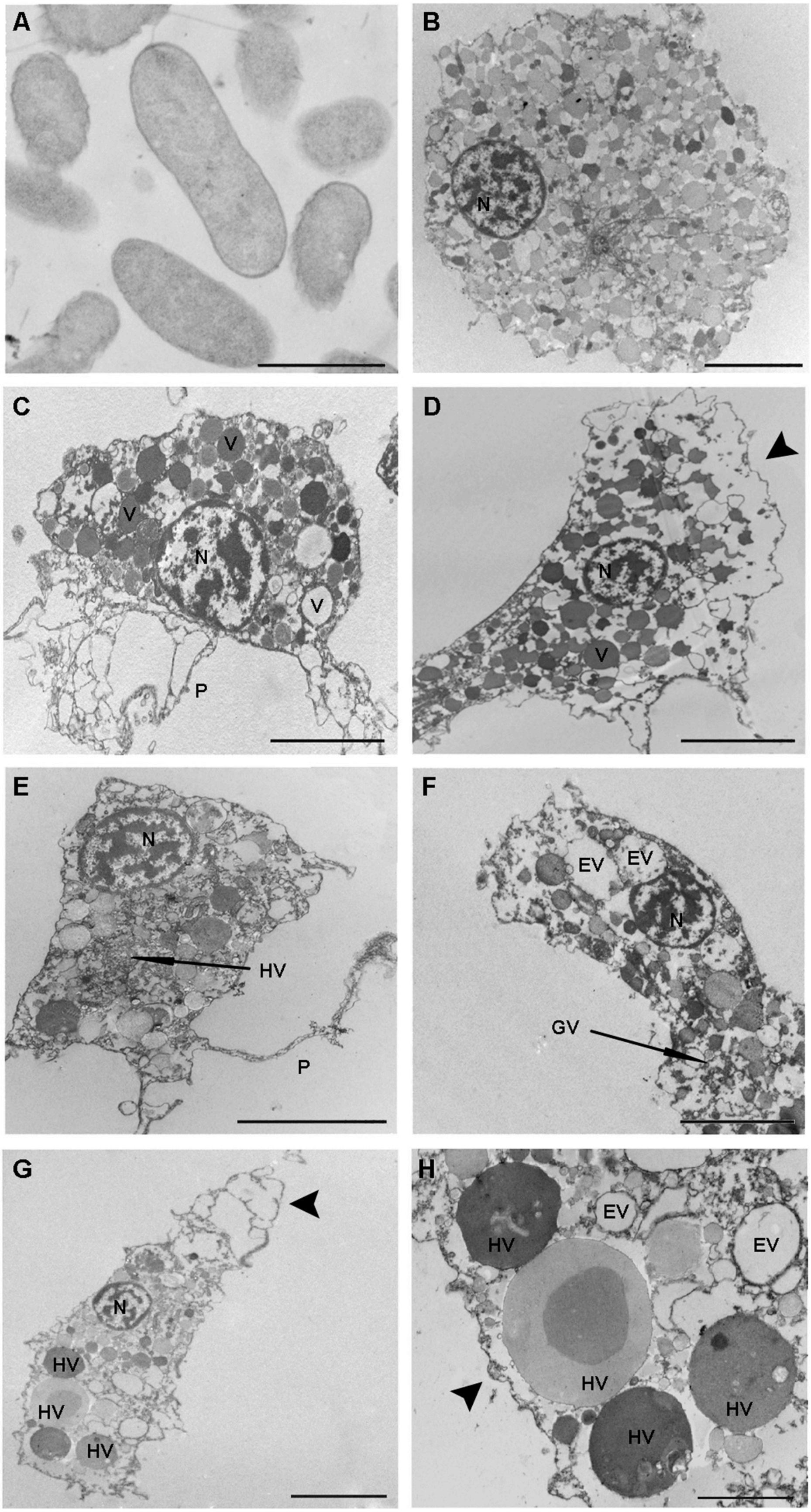

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**Fig. 3. Early *in vitro* effects of *V. coralliilyticus* on the ultrastructure of mussel hemocytes evaluated by TEM.**
Representative images of A) *V. coralliilyticus* before addition to the hemocytes; B) Control hemocyte; C-H), hemocytes incubated with *V. coralliilyticus* ($5 \times 10^6$ CFU/mL) for 5 min (C–D), 15 min (E–F) and 30 min (G–H).
Nuclei (N), pseudopodial extensions (P), vacuoles (V), vesicles with heterogeneous content (HV), vesicles with granular material (GV), empty vesicles (EV
), irregular plasma membrane surfaces (arrowheads).
Scale bars: A) 1 μm; B-G) 5 μm; H) 2.5 μm.

### 3.4. *Effects of V. coralliilyticus on embryo development*

Fertilized eggs were exposed to different concentrations (from $10^1$ to $10^6$ CFU/mL) of *V. coralliilyticus* ATCC BAA-450 in 96-microwell plates, and the percentage of normal D-larvae was evaluated after 48 hpf. The results, reported in Fig. 5, show that *V. coralliilyticus* significantly affected normal larval development, with an $EC_{50}$ value of $5.045 \times 10^3$ CFU/mL (4.599–5.492, 95% CI) (Fig. 5A). The percentage of normal D-larvae was significantly reduced from the lowest concentration tested (from −30% vs controls at 10 CFU/mL) and a dose-dependent effect was observed at increasing concentrations, up to a complete impairment of normal D-larvae development at $10^6$ CFU/mL (−92.8%) ($p < 0.01$).

When the type of effect caused by bacterial challenge was evaluated (Fig. 5B) *V. coralliilyticus* induced a progressive increase in the percentage of malformed embryos. At the highest concentration tested ($10^6$ CFU/mL), the presence of trocophorae/immature D-veligers was also observed, indicating developmental arrest. In Fig. 5C representative images of control embryos and embryos exposed to different concentrations of *V. coralliilyticus* are reported. From the lowest bacterial concentration tested, malformed D-larvae larvae showed irregular shell margins and hinge line and non symmetric valvae. These types of malformation were independent on the bacterial concentration; moreover, at the highest concentration tested, some larvae did not reach the characteristic D-shape.

## 4. Discussion

The present work represents the first investigation on the responses of *M. galloprovincialis* to challenge with the emerging marine pathogen *V. coralliilyticus*. To this aim, the reference ATCC BAA-450 strain isolated from bleached corals near Zanzibar [7], was utilized.

*In vitro* experiments were carried out in the presence of hemolymph serum, in order to simulate the *in vivo* conditions, taking into account also the possible role of soluble hemolymph components, and functional responses of *M. galloprovincialis* hemocytes were evaluated. The results show that *V. coralliilyticus* induced a dose-dependent decrease in hemocyte lysosomal membrane stability that was paralleled by a decrease in the bactericidal activity of whole hemolymph. In particular, at the highest concentration tested ($5 \times 10^7$ CFU/mL) *V. coralliilyticus* was cytotoxic, and no bacterial killing was observed. Moreover, at lower concentrations ($5 \times 10^6$ CFU/mL), no activation of immune parameters was observed in response to *V. coralliilyticus,* except for an extremely rapid extracellular lysozyme release. In these conditions, TEM analysis of hemocytes showed rapid cell damage followed by lysosomal fusion events. No vibrio internalization was observed, indicating no intracellular degradation of bacteria. These results were confirmed *in vivo*, in hemolymph from mussels injected with *V. coralliilyticus* ($5 \times 10^6$ CFU/mL) and sampled after 24 h p.i. In these conditions, *V. coralliilyticus* induced a significant decrease in hemocyte LMS, but did not result in activation of immune parameters; in addition, vibrio challenge even reduced basal ROS production. Accordingly, the results indicate that *V. coralliilyticus* can grow within mussel hemolymph, as shown by the large increase in *Vibrio* counts registered in whole

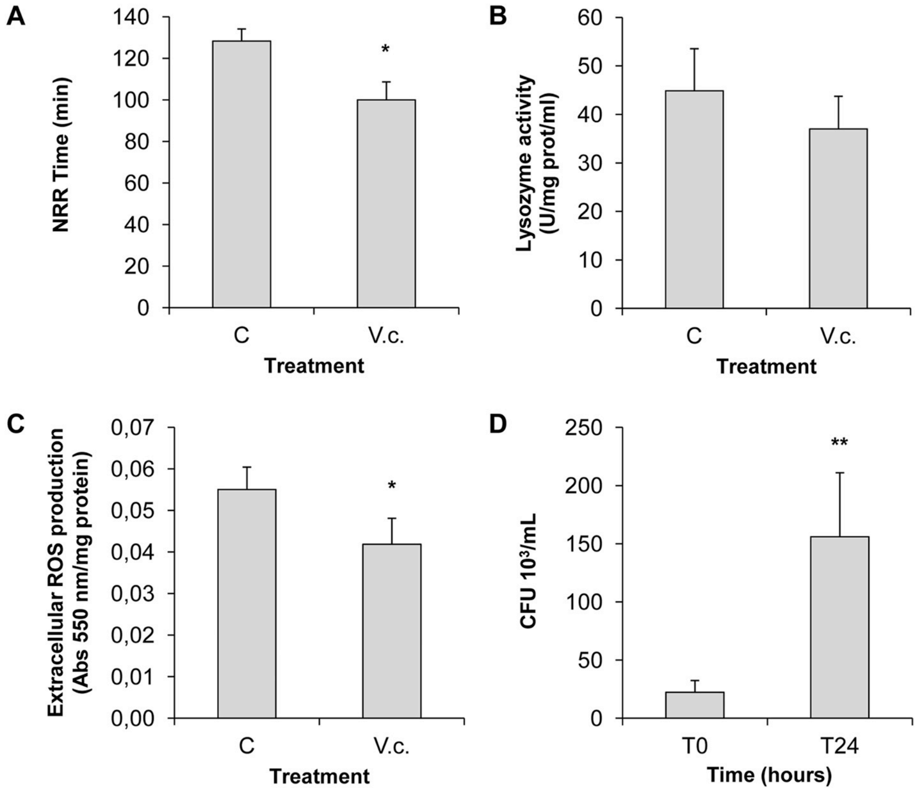

**Fig. 4. *In vivo* effects of *V. coralliilyticus* on hemolymph parameters of *Mytilus* hemocytes. Hemocyte lysosomal membrane stability-LMS.**
(A), serum lysozyme activity (B), ROS production (C) and bacterial cell counts (D) were evaluated in hemolymph sampled from mussels challenged with *V. coralliilyticus* (*V.c.*) at 24 h p.i. Data are the mean ± SD of at least 4 experiments performed in triplicate. Statistical analyses were performed by Mann-Whitney *U* test (* = $p < 0.05$; ** = $p < 0.01$).

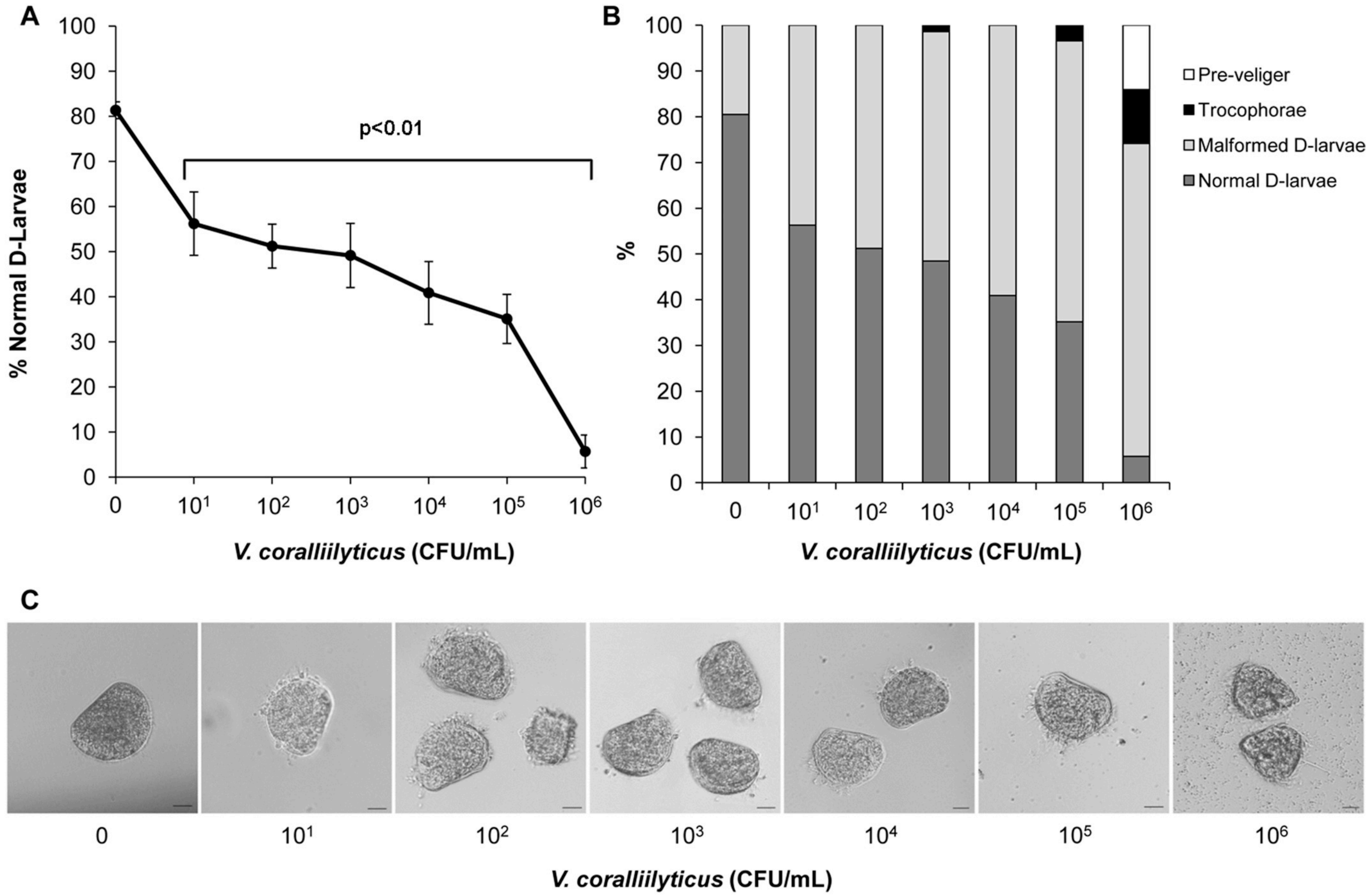

**Fig. 5. Effects of different concentrations of *V. coralliilyticus* on *M. galloprovincialis* normal larval development in the 48 h embriotoxicity assay.**
A) Percentage of normal D-shaped larvae with respect to controls. B) Percentage of normal D-veliger (dark grey), malformed D-veliger (light grey), pre-veligers (white) and trocophorae (black) in each experimental condition. Data, representing the mean ± SD of 4 experiments carried out in 96-multiwell plates (6 replicate wells for each sample), were analyzed by ANOVA plus Tukey's post test ($p < 0.01$). C) Representative images of control embryos and embryos exposed to different concentrations of *V. coralliilyticus*, showing progressive shell malformations, including asymmetric valvae, irregular hinges, externalized velum and, at the highest concentration of bacteria, immature embryos. Bacteria swarming around larvae can be observed at increasing concentrations.

hemolymph samples at 24 h p.i.

In Table 1, the results of the present work are compared with those previously obtained both *in vitro* and *in vivo* with other vibrios (*V. splendidus* LGP32, *V. aestuarianus* 01/032, *V. tapetis* LP2) in similar experimental conditions [18,20]. Overall, although hemocyte responses to *in vitro* challenge with *V. coralliilyticus* appear generally comparable with those of other potential pathogenic vibrios (Table 1, upper panel), differences in individual responses were observed towards different vibrios. In particular, the overall bactericidal activity towards *V. coralliilyticus* was apparently due to extracellular killing (this work) and lower than that observed with *V. aestuarianus,* that was actively degraded within the lysosomal system [18], whereas no bactericidal activity was observed towards *V. splendidus* and *V. tapetis*. Moreover, only *V. tapetis* induced autophagic processes, indicating a protective mechanisms towards damaged cell components [18]. These differences were reflected by *in vivo* data (Table 1, lower panel) showing that mussels were unable to mount an efficient immune response towards *V. coralliilyticus,* this resulting in bacterial growth within the hemolymph. In this respect, the effects of *V. coralliilyticus* were similar to those of the bivalve pathogen *V. splendidus,* and distinct from those of *V. aestuarianus*.

*V. coralliilyticus* also affected early embryo development. Challenge with *V. coralliilyticus* generally resulted in embryo malformations in a wide concentration range and developmental delay at the highest concentration tested. In all experimental conditions, erratic closing of the valves, velum detachment, and bacterial swarming around the embryos were observed, which are clear signs of disease in the larvae [4,24,25]. In both Eastern and Pacific oyster larvae, challenge with *V. coralliilyticus* ATCC BAA-450 for 6 days resulted in mortalities with $LD_{50}$ of 2.1 and $4 \times 10^4$ CFU/mL, respectively [15]. In *C. gigas*, *V. coralliilyticus* also induced a wide range of physiological, enzymatic, biochemical and molecular changes [14]. However, oyster data were obtained in 1–2 weeks old larvae. The results here reported show that *V. coralliilyticus* affects development of mussel embryos at 48 h post fertilization, with significant effect from concentrations as low as 10 CFU/mL. These represent the first data on the effects of *V. coralliilyticus* on early developmental stages of bivalves. In *M. galloprovincialis*, immune capacities arise during mussel development as early as the trocophorae stage (24 hpf). At this developmental stage, gene expression has contributions of maternal origin, but stimulation induces the expression of immune-related genes [26]. However, the present results underline how mussel early embryos are particularly sensitive to *V. coralliilyticus*, and indicate that they are unable to mount a defence response towards this pathogen.

*V. coralliilyticus* possess several virulence mechanisms, including powerful extracellular enzymes that have been linked to direct lysis of coral tissue [8]. Several authors demonstrated that the virulence of some strains is associated with the production of toxins, mainly extracellular metalloprotease (VtpA) and hemolysin (VthA) [27–30]. Furthermore, coral diseases not only depend on the presence of *Vibrio* pathogens and their virulence level, but are also the result of complex interactions between the expression of different bacterial virulence

**Table 1**
Summary of *in vitro* and *in vivo* data on immune responses of *M. galloprovincialis* to challenge with different vibrios.

| *In vitro* | *V. coralliilyticus* ATCC BAA-450[a] | *V. splendidus* LGP32[b] | *V. aestuarianus* 01/032[b] | *V. tapetis* LP2[c] |
|---|---|---|---|---|
| CFU/mL | $5 \times 10^6$ | $10^7$ | $10^7$ | $10^7$ |
| LMS | ↓ | ↓ | ≈ | ↓ |
| Bactericidal activity | ↑ | ≈ | ↑↑ | ≈ |
| Lysozyme activity | ↑ | ↑ | ↑ | ≈ |
| ROS production | ≈ | nd | nd | ↓ |
| NO production | ≈ | nd | nd | ≈ |

| *In vivo* | *V. coralliilyticus* ATCC BAA-450[a] | *V. splendidus* LGP32[b] | *V. aestuarianus* 01/032[b] |
|---|---|---|---|
| CFU/mL | $5 \times 10^6$ | $10^7$ | $10^7$ |
| LMS | ↓ | ↓ | ↓ |
| Bactericidal activity | ≈ | ≈ | ↑ |
| Lysozyme activity | ≈ | ↑ | ↑ |
| ROS production | ↓ | nd | nd |
| NO production | nd | nd | nd |

"nd" = not determined; "≈" = no differences compared to control.
[a] This work.
[b] Balbi et al., 2013 [20].
[c] Balbi et al., 2018 [18].

factors and an increase of seawater temperature or other environmental stresses, as well as the physiological and immune status of the coral host [31].

*Vibrio* species are strongly thermodependent. In particular, for the reference strain of *V. coralliilyticus* ATCC BAA-450 a direct temperature regulation of multiple virulence mechanisms has been demonstrated at 27 °C [32]. *V. coralliilyticus* is able to invade and to lyse the tissue of the coral *Pocillopora damicornis*, one of the most affected organisms, at temperatures higher than 27 °C, while in a temperature range between 24 °C and 26 °C it kills the symbiotic algae of the coral [9]. At temperature below 24 °C is totally avirulent [9,33]. In the present work, all experiments were carried out at the constant temperature of 18 °C, in order to ensure the health and immune status of the mussels. However, even in these conditions, both adult and embryos of *M. galloprovincialis* are apparently unable to mount an efficient immune response towards *V. coralliilyticus*. This results in lysosomal stress in the hemocytes both *in vitro* and *in vivo*, in bacterial growth in the hemolymph of adult mussels challenged *in vivo*, and in malformations in early embryos. Recent data indicate that *in vivo* challenge of the New Zealand Greenshell Mussel *Perna canaliculus* with a *V. coralliilyticus/neptunius*-like isolate induced perturbations of the immune system, oxidative stress, inflammation and metabolic changes at 6 days p.i [34]. Overall, these findings provide a further insight into the potential pathogenic effects of *V. coralliilyticus* in mussels.

In a global warming scenario, an increase in the seawater temperature could promote the proliferation and the potential disease outbreaks associated with *Vibrio* pathogens also in mussels. This is of particular concern in temperate regions such as the Mediterranean sea, where the relative increase in seawater temperature seems to be higher than in tropical areas [31]. Mediterranean strains of *V. coralliilyticus* have been isolated from diseased *P. clavata* colonies collected at Tavolara island (Sardinia, Italy) [16]. Among these, the most virulent strain is TAV24, recently identified as a new genotype of *V. coralliilyticus* by MLST and *vcpA* gene sequencing analyses [35]. The results here reported indicate that the *in vitro* effects of the TAV24 strain on hemocyte lysosomal membrane stability were comparable with those of the reference strain. The responses of *M. galloprovincialis* to challenge with the highly virulent Mediterranean strain require further investigation. Despite the fact that *V. coralliilyticus* appears to be a global bivalve pathogen, there is limited information about its pathogenicity, infection mechanism and/or disease mitigation. Further studies are needed of long term experimental infections of adult mussels to evaluate mortality records. These studies will contribute to understand the potential threat of this vibrio to bivalve aquaculture in the Mediterranean.

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