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Vibrio areninigrae as a pathogenic bacterium in a crustacean

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ABSTRACT

The occurrence of infectious diseases poses a significant threat to the aquaculture industry worldwide. Therefore, characterization of potentially harmful pathogens is one of the most important strategies to control disease outbreaks. In the present study, we investigated for the first time the pathogenicity of two *Vibrio* species, *Vibrio metschnikovii*, a foodborne pathogen that causes fatalities in humans, and *Vibrio areninigrae*, a bacteria isolated from black sand in Korea, using a crustacean model, the signal crayfish *Pacifastacus leniusculus*. Mortality challenges indicated that injection of *V. metschnikovii* (10^8 CFU/crayfish) has a mortality percentage of 22% in crayfish. In contrast, injection of *P. leniusculus* with 10^8 or 10^7 CFU of *V. areninigrae* resulted in 100% mortality within one and two days post-injection, respectively. *V. areninigrae* was successfully re-isolated from hepatopancreas of infected crayfish and caused 100% mortality when reinjected into new healthy crayfish. As a consequence of this infection, histopathological analysis revealed nodule formation in crayfish hepatopancreas, heart, and gills, as well as sloughed cells inside hepatopancreatic tubules and atrophy. Moreover, extracellular crude products (ECP's) were obtained from *V. areninigrae* in order to investigate putative virulence factors. *In vivo* challenges with ECP's caused >90% mortalities within the first 24 h. *In vitro* challenges with ECP's of hemocytes induced cytotoxicity of hemocytes within the first hour of exposure. These findings represent the first report that *V. areninigrae* is a highly pathogenic bacterium that can cause disease in crustaceans. On the contrary, *V. metschnikovii* could not represent a threat for freshwater crayfish.

1. Introduction

Vibriosis are among the most common diseases in the aquaculture industry worldwide. Outbreaks of *Vibrio*-related infections have caused important economic losses in different aquacultured species, including fish (Ina-Salwany et al., 2019), shrimp (Gonzalez-Escalona et al., 2016; Soto-Rodriguez et al., 2015), shellfish (Goudenège et al., 2015), crayfish (Thune et al., 1991) and seahorse (Ina-Salwany et al., 2019).

Vibrio species are Gram-negative, bacillar in shape and motile, and ubiquitous in marine and estuarine ecosystems. This genus is one of the major bacterial species found in aquaculture farms (Cornejo-Granados et al., 2017; Holt et al., 2020), and its presence in freshwater ecosystems has been previously reported (Cornejo-Granados et al., 2018; Dong et al., 2016; Mishra et al., 2010). Due to its pathogenic potential, wide distribution, and range of hosts, *Vibrio* species are considered as opportunistic pathogens (Soumya Haldar, 2012).

The occurrence of opportunistic infections in aquaculture, including vibriosis, depends on the intricate interaction of pathogens, host, and environment (Bass et al., 2019). However, one of the most important elements for pathogen emergence is the evolution of novel strains (Bayliss et al., 2017), and wild aquatic animals and water and sediment bacterial communities are considered to be the main sources of novel pathogens in aquaculture facilities (Bass et al., 2019; Feist et al., 2019).

Up to date, more than 70 species of *Vibrio* are known (Thompson et al., 2004). However, the pathogenic potential of many of them on aquaculture species remains unclarified. Therefore, in the present study, two *Vibrio* sp. species , that we detected by 16S sequencing of *Pacifastacus leniusculus* intestines (data not shown), was investigated for the first time for their possible pathogenicity in freshwater crayfish. These species include *Vibrio metschnikovii* (Lee et al., 1978), which is considered a foodborne pathogen found in seafood worldwide that can cause fatal infections in human patients with comorbidity (Jensen and

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Jellinge, 2014), and *Vibrio areninigrae*, which was isolated for the first time from black sand collected from Jeju Island, Korea (Chang et al., 2008). Koch's postulates were confirmed by reproducing the disease, recovering the isolate from diseased crayfish, confirming the re-isolated to be the same as the injected bacterium with sequencing of 16S rRNA gene and describing the histologic changes induced by this disease, as well as determining putative virulence factors.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Vibrio metschnikovii Gamaleia 1888 strain was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM 19132). This strain was originally isolated from diseased fowl (Lee et al., 1978).

Vibrio areninigrae J74 strain was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM 22054). This strain was originally isolated from black sand collected from Jeju Island, Korea (Chang et al., 2008).

Both *V. metschnikovii* and *V. areninigrae* were grown separately in marine broth (MB) (Difco™) and incubated at 37 °C with agitation (250 rpm) overnight.

In order to obtain *V. metschnikovii* and *V. areninigrae* inoculums, fresh MB was inoculated with the corresponding overnight bacteria culture, and grown to OD 600 = 1.5, equivalent to 5.6×10^{10} colony-forming units (CFU)/mL for *V. metschnikovii* and to 6.5×10^{10} CFU/mL for *V. areninigrae*. Bacteria were collected by centrifugation at 1500*g* for 5 min at room temperature and washed twice with 0.9% NaCl. Both bacteria stocks were 1:10 serial diluted in 0.9% NaCl to obtain 5.6×10^9 and 5.6×10^8 CFU/ml concentrations for *V. metschnikovii* and 6.5×10^9 CFU/mL, 6.5×10^8 6.1×10^7, 6.5×10^6, 6.0×10^5 CFU/ml concentrations for *V. areninigrae*. Dilutions were immediately used for mortality challenge trials. The number of colony-forming units per ml was verified on marine agar (MA) (Difco™) plates.

2.2. Mortality challenge trials

Freshwater crayfish *P. leniusculus* were obtained from Lake Erken in Sweden and maintained in tanks with aerated running tap water at 12 °C. Intermolt, male and healthy crayfish were used in the following experiments. Three days before the bacteria challenge, crayfish were distributed into 8-liter aquaria (3–5 crayfish/tank) with aerated water at 22 °C. Water was renewed one day before the bacterial challenge. Crayfish were tested to be free of *Vibrio* sp. before starting experiments.

The crayfish were injected at the base of the fourth-pair walking leg, with 100 μL of the bacteria dilutions previously prepared. Final amount injected per crayfish were 5.6×10^8 or 5.6×10^7 CFU for *V. metschnikovii*, and 6.5×10^8, 6.5×10^7, 6.1×10^6, 6.5×10^5, 6.0×10^4 CFU for *V. areninigrae*. The control group for each pathogen was injected with 100 μL of 0.9% NaCl. The mortality of the crayfish was registered daily. The experiments lasted seven days and three biological replicates with 3–5 crayfish in each replicate were performed.

2.3. Re-isolation of Vibrio areninigrae from P. leniusculus hepatopancreas

One gram of hepatopancreas obtained from crayfish at 18 h post-infection (hpi) with the *V. areninigrae* strain was homogenized in 1 mL of 0.9% NaCl. After centrifugation of the tube for five seconds, the supernatant was recovered and 1:10 serially diluted in 0.9% NaCl. Then, 100 μL of the 10^{-3} dilution was spread and incubated on MA for 16 h at 37 °C. Colonies showing similar characteristics of pure *V. areninigrae* colonies were re-plated on MA.

In order to test the pathogenicity of these colonies of re-isolated *V. areninigrae* from the hepatopancreas of crayfish, one single colony obtained from hepatopancreas was inoculated in MB and grown to OD 600 = 1.5, equivalent to 4×10^{10} CFU/mL. Bacteria were collected by centrifugation at 1500*g* for 5 min at room temperature and washed twice with 0.9% NaCl. The bacterial stock was 1:10 diluted in 0.9% NaCl to obtain 4×10^9 CFU/mL concentration. The number of CFU/ml was verified on MA plates for this new isolate.

New sets of 3 crayfish per tank were inoculated in the base of the fourth-pair walking leg, with 100 μL of the 4×10^9 CFU/mL dilution of *V. areninigrae* isolated from hepatopancreas, at a final concentration of 4.6×10^8 CFU/crayfish. Controls inoculated with 0.9% NaCl were included. This experiment was repeated 3 times with 3 crayfish each time.

2.4. PCR analysis of the uridylate kinase encoding pyrH and 16S rRNA genes

DNA was extracted from *V. metschnikovii*, *V. areninigrae* J74 strain and *V. areninigrae* re-isolated from hepatopancreas using DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer protocol. Amplification by PCR of the uridylate kinase encoding gene *pyrH* was performed to confirm the presence of *Vibrio* spp., using the primers pyrH-02-R (GTRAABGCNGMYARRTCCA) and pyrH-04-F (ATGASNACBAAYCCWAAACC) (Thompson et al., 2005). Amplification of the 16S rRNA was performed to identify *Vibrio* species, using the primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Fredriksson et al., 2013; Lane, 1991). The reaction mixture of PCR was prepared separately for each set of primers with a final volume of 20 μL containing: 4 μL of 5 X Phusion HF Buffer (Thermo Scientific), 0.2 μL of 10 mM dNTP Mix (Thermo Scientific), 0.2 μL of Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 0.5 μL (0.5 mM) forward primer, 0.5 μL (0.5 mM) reverse primer, 13.6 μL RNase-Free Water and 1 μL of DNA samples (100 ng). DNA isolated from *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Acinetobacter beijerinckii* were included as controls. PCR amplification was performed for *pyrH* as follows: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 2 min 15 s at 58 °C and 1 min 12 s at 72 °C with a final extension of 7 min at 72 °C. PCR conditions for 16S rRNA were: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C with a final extension of 7 min at 72 °C. PCR products for pyrH and for 16S rRNA were resolved on 1.5% agarose gel stained with GelRed Nucleic Acid Stain™ (Biotium).The bands were excised from the gel, purified with GeneJet Gel Extraction kit (Thermo Fisher Scientific) and sequenced by Sanger method with both forward and reverse primers at the KIGene Service (Center for Molecular Medicine, Karolinska University Hospital, Stockholm). Sequences of both *Vibrio areninigrae* (J74) and *Vibrio areninigrae* re-isolated from hepatopancreas were aligned using CLUSTAL 2.1 and blasted against the NCBI data bank (GenBank™) as well as the EZBiocloud system repository.

2.5. Vibrio areninigrae scanning electron microscopy (SEM)

To confirm the structure and morphological similarity of *V. areninigrae* after re-isolation from hepatopancreas, bacteria suspension was prepared from one single colony, growing until 0D 600 of 1.3 in four mL of MB. One mL of bacteria was collected by centrifugation at 1500*g* for 5 min at room temperature and washed twice with 0.9% NaCl. The bacteria pellet was fixed in five mL of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. *V. areninigrae* pure culture from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM 22054) was processed in the same way to confirm the morphology. Both samples were sent to the Microscopy Unit at the Department of Laboratory Medicine, Karolinska Institute, Stockholm for SEM.

From the remaining bacteria stock from hepatopancreas, 1:10 serial dilution in 0.9% NaCl was prepared and 100 μL of 10^{-5} dilution were spread on MA, selective medium Vibrio ChromoSelect Agar (VCSA) (Sigma-Aldrich), and non-selective tryptic soy agar (TSA) to confirm

pure cultures. Agar plates were incubated 37 °C for 36 h.

2.6. Histopathological study of crayfish infected with V. areninigrae

In order to study the progression of the disease, histological analysis of hepatopancreas, heart and gills from crayfish challenged with *V. areninigrae* at 12 and 18 hpi was performed.

A new experiment with the same conditions mentioned above was performed (three aquaria with four crayfish/aquaria). Crayfish from two aquaria were individually injected with 4.6×10^8 CFU, and the remaining group was injected with 0.9% NaCl. Two of four crayfish/group were fixed in Davidson's solution at 12 and 18 h after injection and processed for histological study. Fixed samples were processed, embedded in paraffin, and sectioned (7 μm thin sections) following standard methods (Bell and Lightner, 1988). The sections were stained with haematoxylin and eosin (H&E) and then analyzed by light microscopy. Photographs of the complete digestive tracts were taken at the same time points from one infected and one healthy crayfish. The remaining crayfish were used as mortality control and died approximately at 24 hpi.

2.7. Extracellular products (ECP's) assay

In order to evaluate the toxicity of crude extracellular products (ECP's) from *V. areninigrae*, *in vivo* and *in vitro* assays were performed following the protocol of Jiravanichpaisal et al., (2009). Briefly, one single colony of *V. areninigrae* isolated from hepatopancreas was grown overnight in 10 mL of MB supplemented with 10% crayfish plasma or with no plasma added. The plasma supplementation was performed to evaluate if crayfish components will stimulate *V. areninigrae* secretion of ECP's. Controls of MB with 10% plasma or without plasma that weren't inoculated with *V. areninigrae* were included in the experiments.

After 18 h of incubation at 37 °C with agitation (250 rpm), the cultures were centrifuged at 1500*g* for 5 min at room temperature and the supernatant was separated and filtered through 0.22 μm membrane filter and kept at 4 °C until crayfish injection. Sterility of the ECP's obtained after filtration was confirmed by the spread of 100 μL of ECP's on MA.

2.7.1. In vivo challenge

Groups of four crayfish maintained as mentioned above, were injected with one of the following treatments: 200 μL of ECP's from *V. areninigrae*, 200 μL of ECP's from *V. areninigrae* supplemented with plasma 10%, 200 μL of MB or 200 μL of MB supplemented with plasma 10%. The experiment was repeated 3 times with 4 crayfish each time. Statistical analysis of the survival obtained was evaluated with the Log-rank (Mantel-Cox) Test.

2.7.2. In vitro challenge

Two mL of hemolymph were obtained individually from healthy intermolt crayfish and immediately mixed with the same volume of anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) (Söderhäll and Smith, 1983). The hemocytes were then separated from plasma by centrifugation at 900*g* for 10 min at 4 °C and washed two times with 0.15 M NaCl. Total hemocyte count was determined using a hemocytometer. Hemocytes were cultured in 96-well plates at a density of 1×10^5 cells/well in 0.15 M NaCl at room temperature (22 °C), with one of the following treatments: 50 μL of NaCl 0.15 M, 50 μL of MB, 50 μL of ECP's or 50 μL of ECP's supplemented with plasma 10%. Morphology and cytotoxicity were evaluated after one hour using trypan blue 4%. Three experimental biological replicates were performed.

Fig. 1. Photographs of the gastro-intestinal tracts (stomach, hepatopancreas and intestine) from *P. leniusculus* injected with 0.9% NaCl (A), 4.6×10^8 CFU of *V. areninigrae* at 12 hpi (B) and 18 hpi (C). Bar scale = 1 cm.

3. Results

3.1. Vibrio metschnikovii is weakly pathogenic to crayfish

Crayfish injected with *V. metschnikovii* at concentrations of 5.6×10^8 or 5.6×10^7 CFU did not show any sign of disease. The animals stayed active during the experiment (seven days). Crayfish injected with 5.6×10^8 had a survival rate of 78% at the end of the experiment; only two animals died two days post-infection (dpi). Crayfish injected with 5.6×10^7 CFU remained active and had a survival rate of 100% at the end of the experiment. Crayfish injected with 0.9% NaCl remained active and did not die during the duration of the experiment. Further characterization of infection with this pathogen was not performed since the mortality results suggested it doesn't represent an important risk for crayfish.

3.2. Vibrio areninigrae pathogenicity and gross signs of infection

Crayfish infected with *V.areninigrae* at a concentration of 4.6×10^8 CFU became lethargic already at 12 hpi and this continued as the infection developed. Hepatopancreas developed discoloration and an aqueous consistency as the infection progressed. The intestine was empty in the infected crayfish, indicating some problems with feeding or digestion (Fig. 1).

We tested the survival rate of crayfish injected with *V. areninigrae* J74 strain at different doses, and Fig. 2 shows a dose-dependent mortality rate of crayfish injected with *V. areninigrae* J74 strain and *V. areninigrae* re-isolated from the hepatopancreas of previously *V. areninigrae* infected animals. Crayfish that received 6.5×10^8 CFU became lethargic at 12 hpi and presented a median survival of one day, while the crayfish group injected with 6.5×10^7 CFU had a median survival of two days. In contrast, 83% of the crayfish injected with 6.1×10^6 CFU survived at the end of the experiment, with two animals dying at 3.5 dpi. Crayfish injected with 6.5×10^5 and 6.0×10^4 CFU, as well as the control group, all survived at the end of the experiment. To confirm the pathogenicity, we re-isolated bacteria from hepatopancreas of infected and clearly affected animals. The crayfish injected with 4.6×10^8 CFU of these bacteria re-isolated from hepatopancreas had a median survival of one day, in accordance with the mortality rate obtained in the first infection experiment. Control crayfish injected with 0.9% NaCl remained active and didn't show mortalities during the seven days of the experiment.

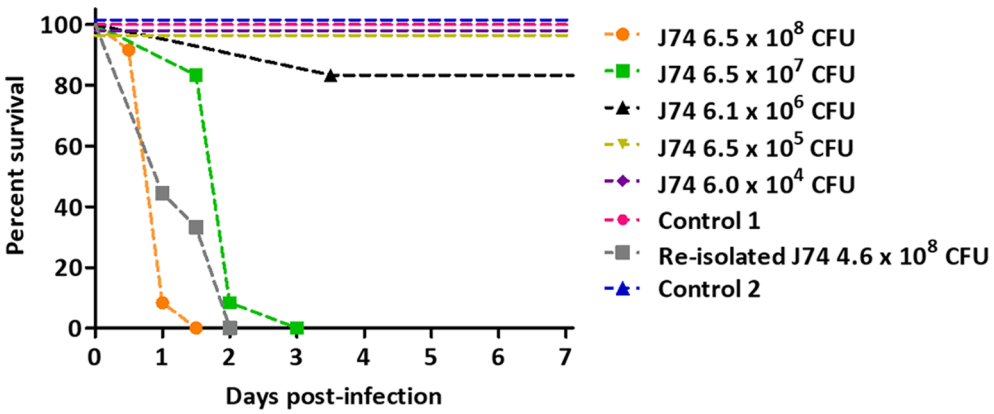

Fig. 2. Percent survival of *P. leniusculus* after injection with *V. areninigrae* (strain J74) at different doses, and *P. leniusculus* injected with 0.9% NaCl (Control 1). Percent survival of crayfish *P. leniusculus* after infection with re-isolated bacteria from hepatopancreas of previously *V. areninigrae* infected animals, then confirmed to be *V. areninigrae* (Re-isolated J74) by sequencing, and P. leniusculus injected with 0.9% NaCl (Control 2). At least 3 animals were included per treatment and the experiments were repeated three times.

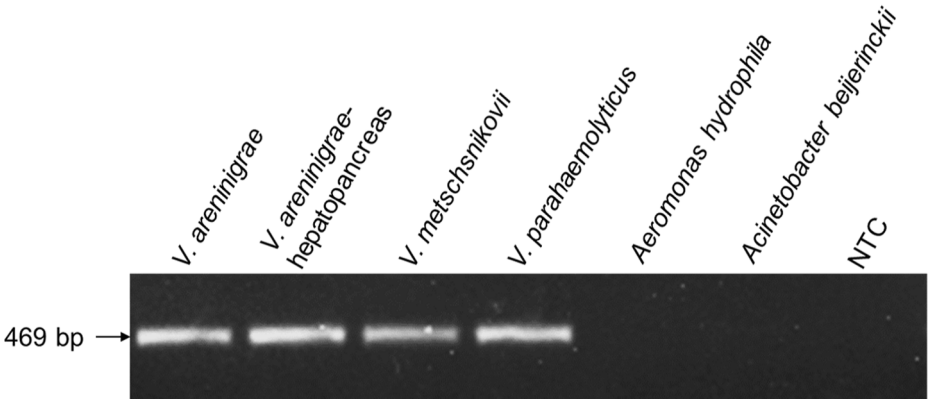

Fig. 3. Detection of the uridylate kinase encoding gene pyrH by PCR from *V. areninigrae* J74 strain, *V. areninigrae* re-isolated from hepatopancreas, *V. metschnikovii*, *V. parahaemolyticus*, *Aeromonas hydrophila* and *Acinetobacter beijerinckii*. NTC: No template control.

3.3. Identification of Vibrio areninigrae re-isolated from infected crayfish by molecular methods

Amplification and sequencing of the *pyrH* and 16S rRNA genes using the DNA extracted from *Vibrio* areninigrae (J74 strain) and *Vibrio areninigrae* re-isolated from infected crayfish were used to confirm the identity of the species recovered. As shown in Fig. 3 the isolated *Vibrio* from crayfish hepatopancreas after injection (*V. areninigrae*-hepatopancreas), was confirmed by PCR with the amplification of the gene *pyrH*. Aligning with CLUSTAL 2.1 of the sequences of the injected *V. areninigrae* J74 strain (original), with the obtained sequence of the re-isolated strain showed 100% similarity with 16S as well as the pyrH primers (Supplementary figures S1-S2). The sequences obtained with 16S rRNA gene showed 100% identity with *Vibrio areninigrae* (J74) when analyzed with NCBI (GenBank™) and EZBiocloud data banks confirming *V. areninigrae* as the causative agent of the mortalities. So far, there are no reference sequences for *pyrH* of *V. areninigrae* in any databases, and the sequences obtained with degenerate primers for pyrH showed the highest similarity percentage with *Photobacterium swingsii* (99%) using BLAST at NCBI (GenBank™), while the 16S sequence showed 96% similarity with this species.

3.4. Morphological study of Vibrio areninigrae using SEM

SEM micrographs showed pure culture from the microbe collection of *V. areninigrae* J74 (Fig. 4A and C), as well as pure culture re-isolated from the hepatopancreas of moribund crayfish (Fig. 4B and D). The cells are slightly curved, rod-shaped, and the length varies between 1.0 and 3.0 μm. Fig. 4C shows asymmetric division or 'budding' of *V. areninigrae*. *Vibrio areninigrae* grows exclusively in marine agar. No growth was observed in TSA or selective medium VCSA.

3.5. Histopathological study of V. areninigrae challenged crayfish

We then investigated the histopathology of *V. areninigrae* infected crayfish in order to study the manifestations of disease on the animal. In Figs. 5-7 histopathological changes at 12 and 18 hpi with *V. areninigrae* in hepatopancreas, heart, and gills are shown. After 12 h of infection with *V. areninigrae*, crayfish showed cellular immune response (Cerenius et al., 2008, Cerenius and Söderhäll, 2018) such as nodule formation in hepatopancreas (Fig. 5B), heart (Fig. 6B), and gills (Fig. 7B). Later, after 18 h of infection, the crayfish hepatopancreas showed atrophy, pyknotic and sloughed cells inside hepatopancreatic tubules (Fig. 5C), as well as bacteria clumps (Fig. 5D). In addition, nodule formation was observed in heart (Fig. 6C and D), and cell aggregation and pyknotic cells in gills (Fig. 7C and D). Control crayfish didn't show any pathological changes (Figs. 5A, 6A and 7A).

3.6. Extracellular products (ECP's) toxicity

In order to elucidate whether the ECP's produced from *V. areninigrae* play a role in its pathogenicity, ECP's were prepared and tested in both *in vivo* and *in vitro* assays.

3.6.1. In vivo challenge

Injection of ECP's from *V. areninigrae* resulted in mortality of crayfish. At the end of the experiment on day 7, survival from crayfish group injected with ECP's was 8.3% (median survival time 0.89 dpi), and for the crayfish group injected with ECP's supplemented with plasma was 16.6% (median survival time 1.8 dpi) (Fig. 8). No significant difference

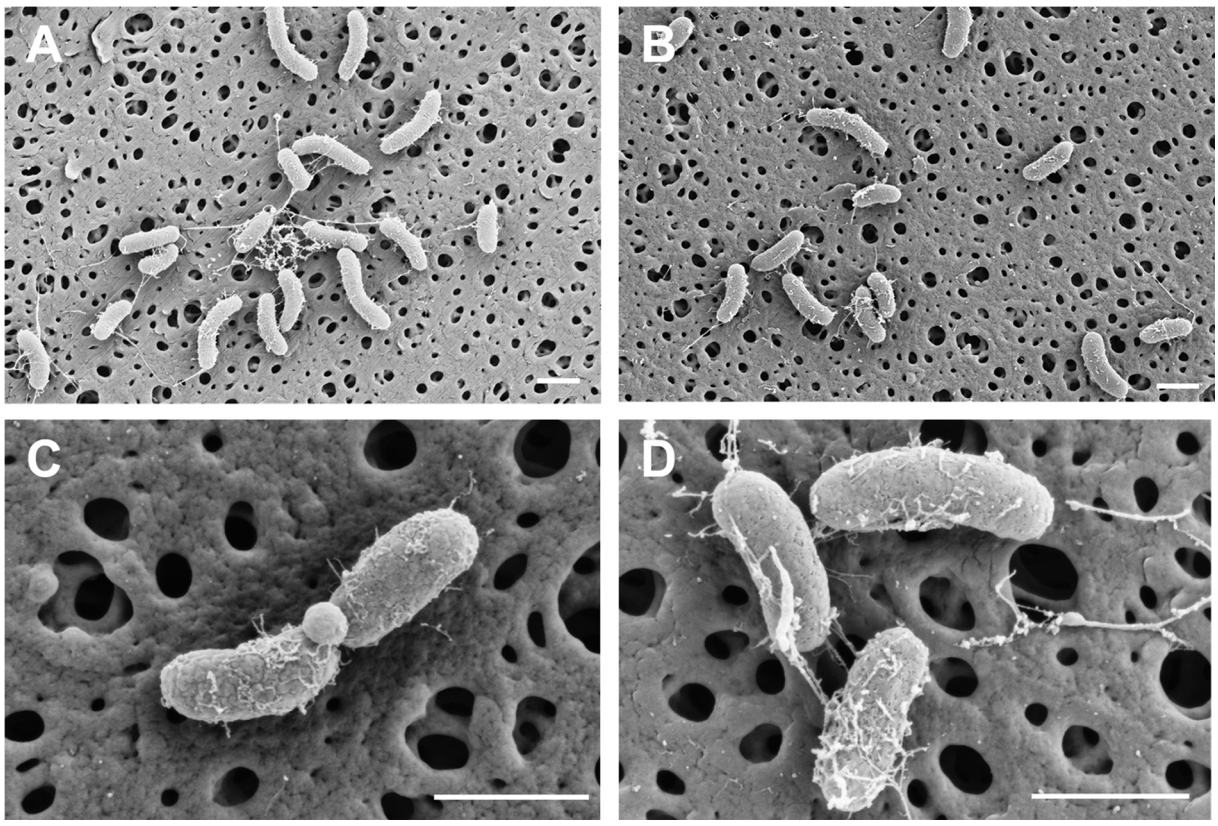

Fig. 4. SEM images of *Vibrio areninigrae*. (A) and (C) *V. areninigrae* J74 strain (DSM 19132); (B) and (D) *V. areninigrae* re-isolated from the hepatopancreas of moribund crayfish after injection; (C) *V. areninigrae* budding. Length of cells 1.0–3.0 μm. Bar scales = 1 μm.

was obtained from the comparison of these two groups ($P = 0.3738$). Control groups of crayfish remained active and showed no mortalities during the experiment (Fig. 8). No bacterial growth was observed in the MA after spreading with ECP's, which confirmed sterility of these samples.

3.6.2. *In vitro challenge*

We then tested the effect of ECP's on isolated hemocytes *in vitro*, and Fig. 9 shows the result of ECP's effect on total hemocytes maintained with 0.15 NaCl after one-hour incubation in the different treatments. Hemocytes from control (0.15 M NaCl) were viable and maintained normal shape (Fig. 9A), while hemocytes inoculated with MB remained viable and showed slight agglutination (Fig. 9B). Hemocytes inoculated with ECP's (Fig. 9C) and ECP's supplemented with plasma 10% (Fig. 9D) showed more than 95% cell death as judged by trypan blue staining one hour after incubation with *V. areninigrae* ECPs.

4. Discussion

Two species of *Vibrio* sp. namely *V.metschnikovii* and *V. areninigrae* were detected when we performed 16S sequencing of *P. leniusculus* intestines (data not shown) and that is the reason why they were tested for their potential as pathogens for a crustacean, the freshwater crayfish *P. leniusculus*. Therefore, crayfish were challenged via injection with these *Vibrio* species to assess the pathogenic potential of these bacteria for the first time in any crustacean.

Our results showed that the overall pathogenicity of *Vibrio metschnikovii* can be considered weak and this species doesn't represent a threat for *P. leniusculus* in terms of mortality. Crayfish had a mortality of about 22% at two days post-injection with high doses of *V. metschnikovii*. Similar results were obtained before in *P. leniusculus* with other enteric bacteria, including *Citrobacter* sp., *Acinetobacter* sp., and *Pseudomonas* sp., (Jiravanichpaisal et al., 2009), where the use of large inoculums failed to cause death, presumably because of an effective immune response. This is a notable result since, although *V. metschnikovii* is widely distributed in aquatic species, including scallop, bird clam, oyster, shrimps, lobster, crab and fish (Antunes et al., 2010; Farmer et al., 1988; Lee et al., 1978), and its presence has been reported as part of microbial communities in shrimp ponds (Sung et al., 2001), information regarding its pathogenicity is not completely robust. For example, Aguirre-Guzmán et al., (2004) considered *V. metschnikovii* a as non-pathogenic bacterium for shrimp, but it is not clear which methodological approach the authors used to reach this conclusion.

Moreover, the results reported herein have implications for public health, since it is shown that *P. leniusculus* acts as a carrier of *V. metschnikovii*, which can cause human disease including gastrointestinal tract disease (Dalsgaard et al., 1996), pneumonia (Wallet et al., 2005) and, in comorbidity cases, septicemia, cardiac arrest and fatalities (Jensen and Jellinge, 2014; Linde et al., 2004). In addition, consumption of cooked crayfish has been associated with vibriosis infection (Bean et al., 1998) and incidence of ca 10% of *V. metschnikovii* has been previously reported in seafood markets (Elhadi et al., 2004).

Regarding *V. areninigrae*, we successfully recovered *V. areninigrae* from crayfish previously infected, as the molecular analysis with 16S rRNA confirmed. However, it is important to mention that exact identification with *pyr*H gene sequencing is not possible for this *Vibrio* species using degenerate primers. This is due to a lack of information available in databases, and although detection of *Vibrio* sp. using *pyrH* is widely used, it is important to consider that the ranges of intra and interspecificity sequence similarity are lower than, for example 16S rRNA (Pascual et al., 2010).

Clinical signs observed in infected crayfish with *V. areninigrae* included typical vibriosis signs i.e. lethargy, empty gut, pale and aqueous hepatopancreas (Soto-Rodriguez et al., 2015), and injection of

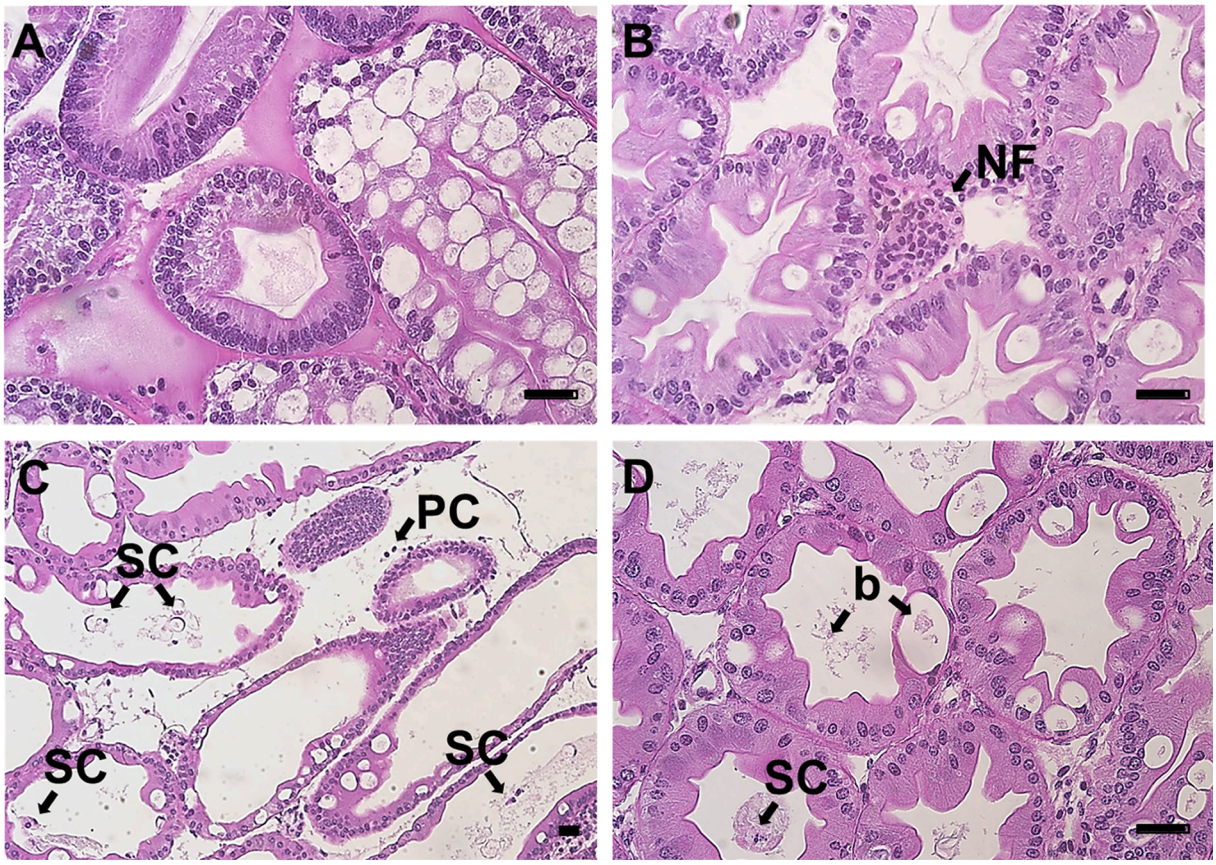

Fig. 5. Histopathological analysis of hepatopancreas from crayfish injected with 4.6×10^8 CFU of *V. areninigrae*. Control group showed no pathological changes (A). After 12 h, early stage of nodule formation (NF) was observed (B). After 18 h atrophy, presence of pyknotic cells (PC), sloughed cells (SC), and bacteria (b) inside the hepatopancreatic tubules was observed (C and D). Bar scales = 50 μm.

10^8 or 10^7 CFU resulted in 100% mortality of crayfish within one and two days, respectively. Moreover, after receiving an injection with *V. areninigrae* filtrate (ECP's) the mortality of crayfish was high (>80%) and in a very short time (1–2 days). These results suggest that *V. areninigrae* produces extracellular toxins which are part of the virulence factors of this bacterial species. Extracellular products from different *Vibrio* species and strains have been extensively studied before, including adhesins, alkaline proteases, chitinases, cysteine proteases, hemolysins, metalloproteases, serine proteases, type III (T3SS) and type VI (T6SS) secretion systems and ureases (Aguirre-Guzmán et al., 2004; Beshiru and Igbinosa, 2018; Igbinosa, 2016; Labreuche et al., 2017; Le Roux et al., 2015; Li et al., 2019; Sirikharin et al., 2015; Zhang et al., 2020). It has also been demonstrated that pathogenicity of *Vibrio* is the result of a complex combination of multiple virulence factors (Li et al., 2019; Sirikharin et al., 2015). Although the objective of this study wasn't to characterize the toxins, but to address the pathogenic potential of this bacterium, our results also showed a cytotoxic effect of *V. areninigrae* ECP's towards *P. leniusculus* hemocytes in an *in vitro* study. Cells became unviable one hour after exposure to ECPs. This confirms that mortalities herein observed were not the result of bacterial multiplication, but more likely caused by toxins.

Furthermore, the most distinct result from histopathological analysis was early nodule formation in hepatopancreas, heart, and gills of *V. areninigrae* infected crayfish after 12 h-infection. This suggests that even if crayfish were able to mount cellular immune reactions, especially during the first hours of infection, it is very likely that as time progressed, the toxin production caused animal death. Moreover, hepatopancreas showed detachment of tubular epithelial cells or cell sloughing, which is considered a pathognomonic lesion of *Vibrio*-related diseases like acute hepatopancreatic necrosis disease (AHPND) (Angthong et al., 2017; Dhar et al., 2019; Sirikharin et al., 2015; Soto-Rodriguez et al., 2015; Velázquez-Lizárraga et al., 2019) and *V. harveyi* infection (Zhang et al., 2020), and that is known to be caused by *Vibrio* toxins (Sirikharin et al., 2015).

It is worth mentioning that until now, the only information regarding *V. areninigrae* available is related to taxonomical identification and biochemical analysis (Chang et al., 2008; Rim Kang et al., 2015), and since this bacterium was originally isolated from an active aquacultural zone, Jeju Island (FAO, 2016; Yun et al., 2015), elucidation of *V. areninigrae* pathogenic potential is of utmost importance for the local shrimp industry. Nonetheless, it is worth to mention that freshwater crustaceans could present similar mortalities rates to microbial pathogens as marine crustaceans (Longshaw, 2011).

Vibriosis outbreaks from environmental reservoirs depend upon the specific ecology, disease dynamics, and etiology (Holt et al., 2020). However, characterization of pathogens and the virulence factors have proven to provide valuable information that can be used to develop prevention and mitigation strategies, contributing to strengthening the sustainability of crustacean farming.

5. Conclusions

Our results show that *V. areninigrae* is a highly pathogenic bacterium for crayfish *P. leniusculus* and that the production of virulence factors is responsible for crayfish death. Koch's postulates were fulfilled during the characterization of the disease. *V. metschnikovii*, however, is a weakly-pathogenic bacterium for this crustacean.

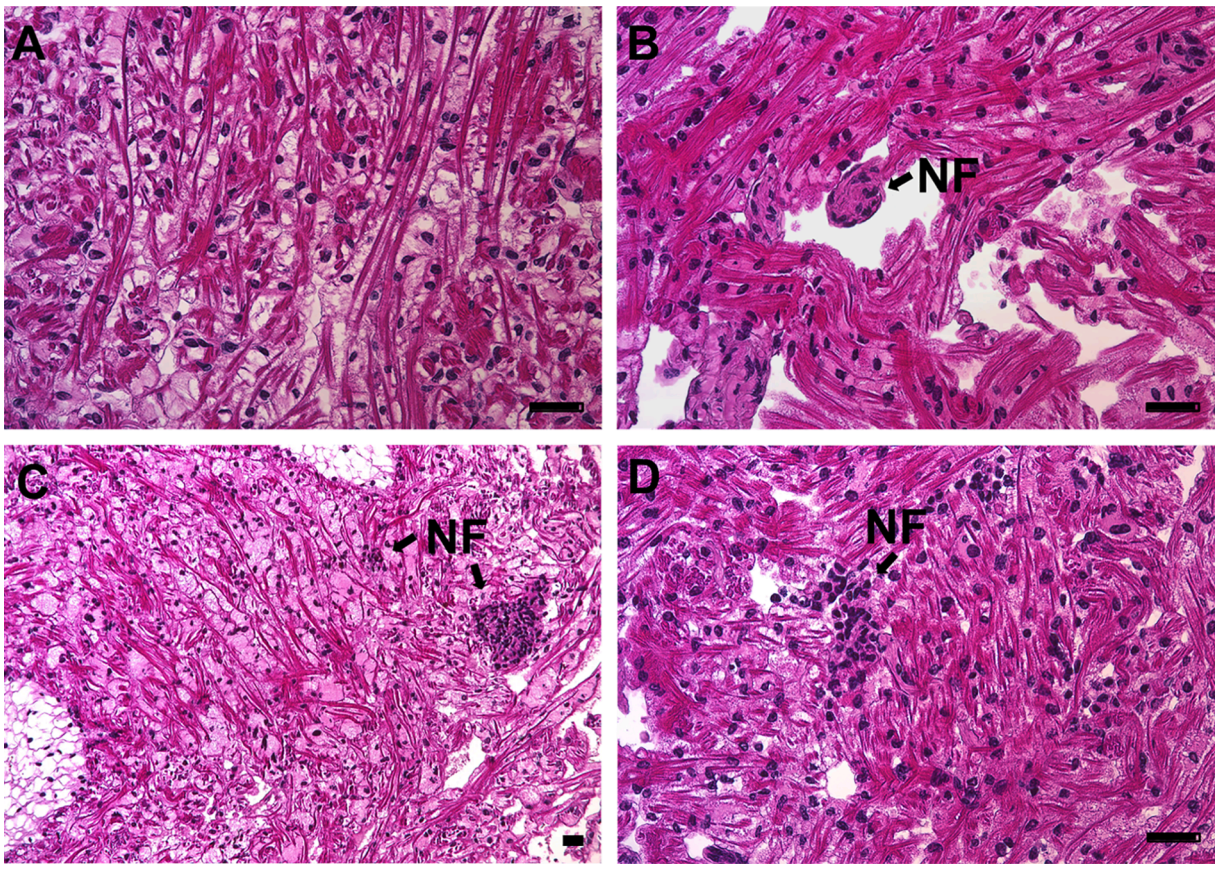

Fig. 6. Histopathological analysis of heart from crayfish injected with 4.6×10^8 CFU of *V. areninigrae*. Control group showed no pathological changes (A). After 12 and 18 h, early stage of nodule formation (NF) was observed (B, C and D). Bar scales = 50 μm.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2020.107517.

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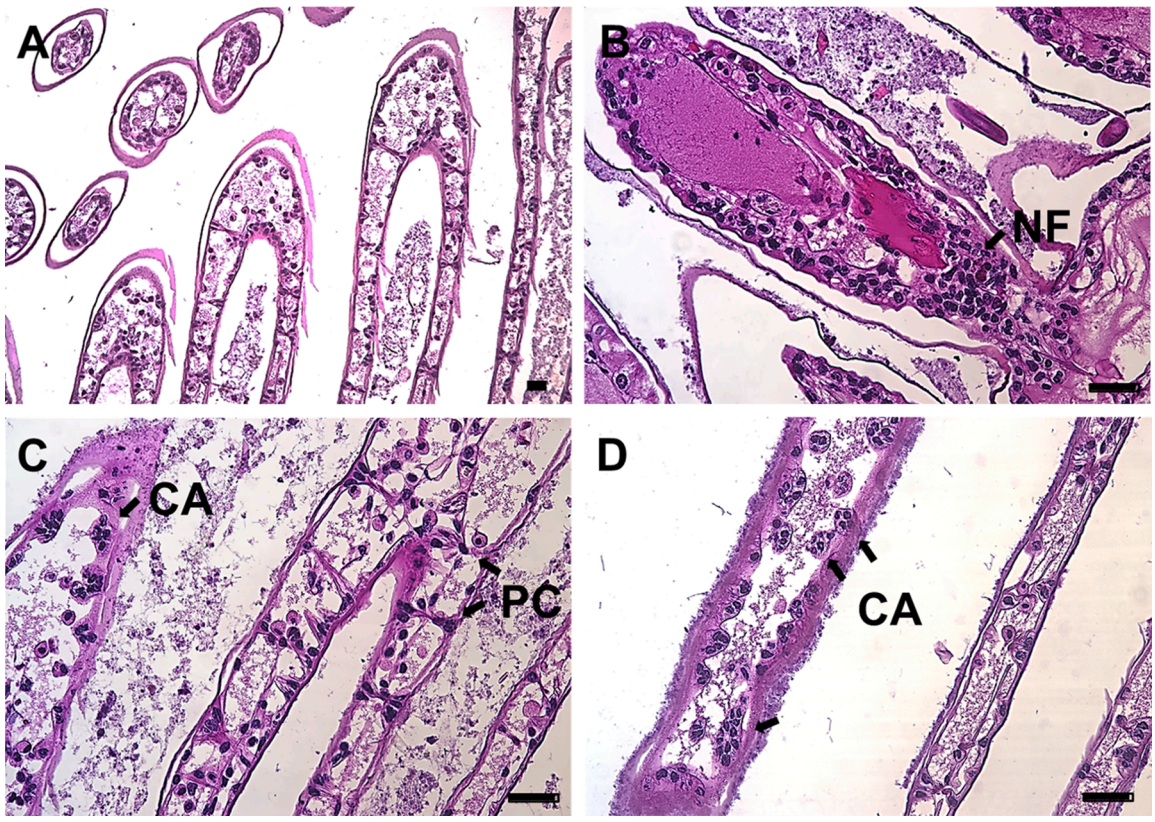

Fig. 7. Histopathological analysis of gills from crayfish injected with 4.6×10^8 CFU of *V. areninigrae*. Control group showed no pathological changes (A). After 12 h early stage of nodule formation (NF) was observed (B). After 18 h cell aggregation (CA) and the presence of pyknotic cells (PC) were observed (C and D). Bar scales = 50 μm.

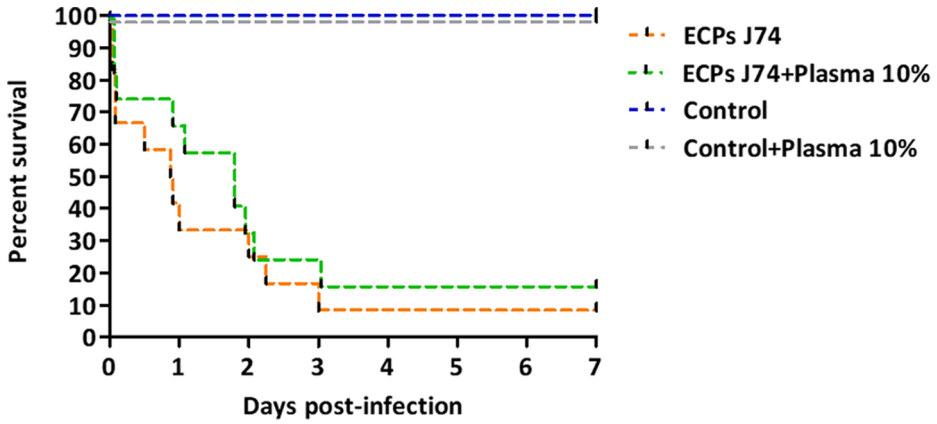

Fig. 8. Percent survival of *P. leniusculus* after injection with *V. areninigrae* extracellular products (ECP's). Median survival 0.89 dpi for ECP's group and 1.8 dpi for ECP's supplemented with plasma 10%. No significant difference between ECP's treatments was obtained (P = 0.3738). Control groups did not show any mortalities. Four animals were included per treatment and the experiments were repeated three times.

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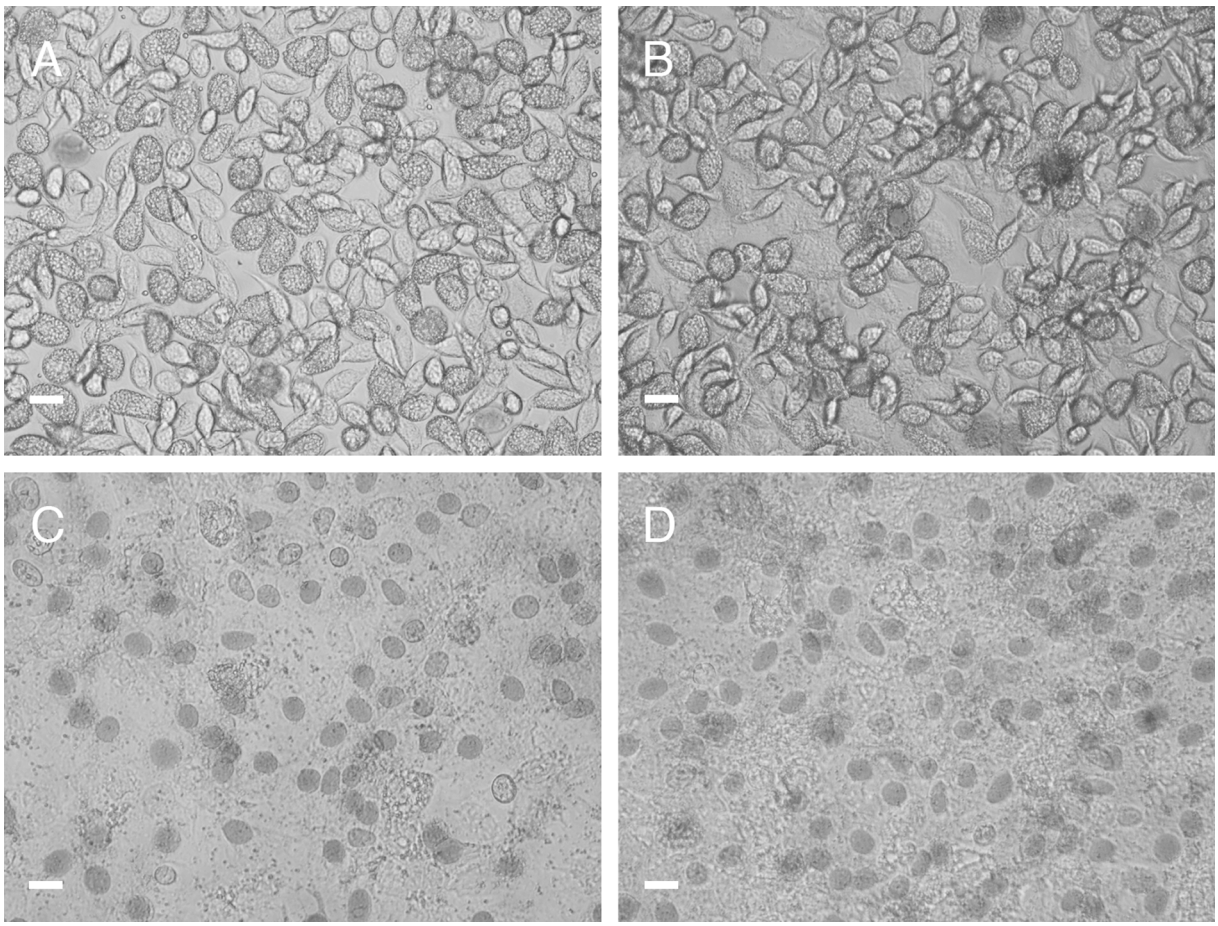

Fig. 9. ECP's cytotoxicity in *in vitro* assay of total hemocytes. Cells were maintained in NaCl 0.15 M at room temperature (22 °C) and treated with: (A) NaCl 0.15 M, (B) marine broth, (C) *V. areninigrae* ECP's or (D) *V. areninigrae* ECP's supplemented with plasma (10% final concentration). After incubation for one hour, trypan blue 4% was added. Hemocytes incubated with NaCl (A) maintained viability. Hemocytes incubated with marine broth (B) maintained viability and showed agglutination. Hemocytes incubated with ECP's (C) and ECP's supplemented with plasma (10% final concentration) (D) showed cytoplasmic vacuolization and cellular death after incubation for one hour. Bar scale = 20 μm.

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