Fish & Shellfish Immunology
March 2023, Volume 134 Pages 108622 (16p.)
https://doi.org/10.1016/j.fsi.2023.108622
https://archimer.ifremer.fr/doc/00828/93986/

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The first use of LC-MS/MS proteomic approach in the brown mussel *Perna perna* after bacterial challenge: Searching for key proteins on immune response

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Abstract :

The brown mussel Perna perna is a valuable fishing resource, primarily in tropical and subtropical coastal regions. Because of their filter-feeding habits, mussels are directly exposed to bacteria in the water column. Escherichia coli (EC) and Salmonella enterica (SE) inhabit human guts and reach the marine environment through anthropogenic sources, such as sewage. Vibrio parahaemolyticus (VP) is indigenous to coastal ecosystems but can be harmful to shellfish. In this study, we aimed to assess the protein profile of the hepatopancreas of P. perna mussel challenged by introduced - E. coli and S. enterica - and indigenous marine bacteria - V. parahaemolyticus. Bacterial-challenge groups were compared with non-injected (NC) and injected control (IC) - that consisted in mussels not challenged and mussels injected with sterile PBS-NaCl, respectively. Through LC-MS/MS proteomic analysis, 3805 proteins were found in the hepatopancreas of P. perna. From the total, 597 were significantly different among conditions. Mussels injected with VP presented 343 proteins downregulated compared with all the other conditions, suggesting that VP suppresses their immune response. Particularly, 31 altered proteins - upregulated or downregulated - for one or more challenge groups (EC, SE, and VP) compared with controls (NC and IC) are discussed in detail in the paper. For the three tested bacteria, significantly different proteins were found to perform critical roles in immune response at all levels, namely: recognition and signal

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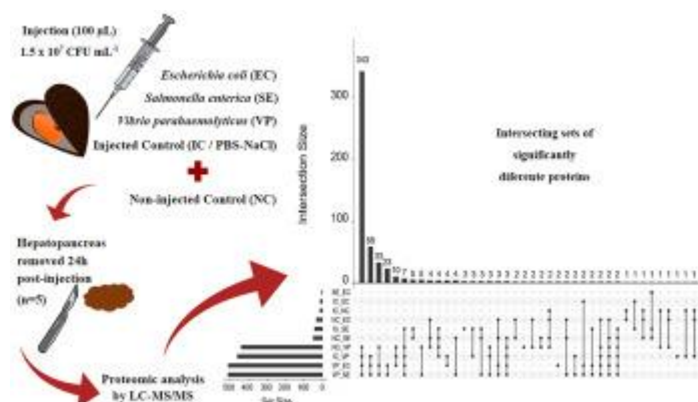
transduction; transcription; RNA processing; translation and protein processing; secretion; and humoral effectors. This is the first shotgun proteomic study in P. perna mussel, therefore providing an overview of the protein profile of the mussel hepatopancreas, focused on the immune response against bacteria. Hence, it is possible to understand the immune-bacteria relationship at molecular levels better. This knowledge can support the development of strategies and tools to be applied to coastal marine resource management and contribute to the sustainability of coastal systems.

Graphical abstract



Highlights

► A total of 3805 proteins were found in the hepatopancreas of *P. perna* mussel. ► *Vibrio parahaemolyticus* seems to be virulent and suppresses *P. perna* immune response. ► *E. coli* and *S. enterica* triggered a general immune response against bacteria. ► Proteins altered in response to all challenge-bacteria were vital for immune defense. ► This study points out critical proteins against bacteria, promising for biotechnology.

Keywords : Escherichia coli, Hepatopancreas, Immune-related proteins, LC-MS, MS, Perna perna, Protein profile, Salmonella enterica, Shellfish, Vibrio parahaemolyticus

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1. Introduction

Coastal pollution is a concern worldwide and is often concentrated along densely-populated areas in low- and middle-income countries (Landrigan et al., 2020; Tuholske et al., 2021). Continental runoff and sewage introduce excessive nutrients and pathogenic bacteria in the coastal waters, therefore altering the local microbial communities and, consequently, the ecological dynamics (Buccheri et al., 2019; Tuholske et al., 2021; Young et al., 2016). Additionally, anthropogenic pollution, not to mention climate change, can increase the abundance of introduced bacteria in the seawater and also alter the geographic ranges of indigenous marine pathogens (Burge et al., 2013; Cavicchioli et al., 2019; Chen et al., 2019).

Escherichia coli and *Salmonella enterica* belong to the Enterobacteriaceae family and reach the marine environment through anthropogenic sources, such as sewage (Buccheri et al., 2019). *Escherichia coli* naturally inhabit human and other warm-blooded animal guts (Gomes et al., 2016; Jang et al., 2017; Yang et al., 2017). Since *E. coli* is discharged in the feces, its presence and abundance in the environment indicate the fecal contamination degree, as well as the presence of other intestinal pathogens (Gomes et al., 2016; Jang et al., 2017; Yang et al., 2017).

As part of the human microbiota, *E. coli* rarely induces disease in healthy individuals. However, some strains are known as diarrheagenic, because they cause diarrhea or extraintestinal diseases (Gomes et al., 2016; Jang et al., 2017; Yang et al., 2017). Within diarrheagenic pathotypes, Enteropathogenic *E. coli* (EPEC) strains are described as those that cause diarrhea, render attaching and effacing lesions on the intestinal epithelium, and cannot produce Shiga toxins and heat-labile or heat-stable enterotoxins (Gomes et al., 2016; Jang et al., 2017; Yang et al., 2017).

Similarly, *Salmonella* spp. transmission can also be related to fecal contamination by the ingestion of contaminated water or food (Balasubramanian et al., 2019; Ferrari et al., 2019). Depending on their specificity to human hosts, *Salmonella enterica* serovars are categorized as typhoid - TS or nontyphoid – NTS (Balasubramanian et al., 2019; Ferrari et al., 2019). NTS serovars, such as *S. typhimurium,* infect a wide range of hosts and usually provoke a self-limiting diarrheal disease in humans (Balasubramanian et al., 2019; Ferrari et al., 2019).

In contrast to *E. coli* and *S. enterica*, *Vibrio parahaemolyticus* is indigenous to coastal and estuarine ecosystems being associated with several marine organisms - e.g., fish, mollusks, and crustaceans (Bonnin-Jusserand et al., 2019; Ghenem et al., 2017). *Vibrio parahaemolyticus* together with *V. cholerae* and *V. vulnificus* comprise the three most important human pathogenic strains in the genus (Bonnin-Jusserand et al., 2019; Ghenem et al., 2017). This first species is a relevant causal agent of human gastroenteritis associated with the consumption of marine foods (Bonnin-Jusserand et al., 2019; Ghenem et al., 2017).

Because of their filter-feeding habits, bivalve mollusks are directly in contact with bacteria suspended in the water column, either indigenous or introduced (Silva dos Santos et al., 2018). To thrive in a bacteria-rich environment, bivalves count with a robust innate immune system based on cellular and humoral responses (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). The main cellular effectors in the mussel hemolymph are known as hemocytes. These circulating cells act on phagocytosis, encapsulation, and infiltration to repair and defend damaged or infected tissues (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). Additionally, hemocytes can release

reactive oxygen/nitrogen species (ROS/RNS), antimicrobial peptides (AMPs), and lysosomal enzymes as defense mechanisms (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020).

On the other response front, humoral factors in the hemolymph and tissues act synced with hemocytes (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). The first step to initiating an immune reaction against pathogens is the detection and recognition (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). Hence, evolutionarily conserved molecules - pattern recognition receptors (PRRs) - identify specific markers in the pathogens, known as pathogen-associated molecular patterns (PAMPs) (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). The main PRRs are toll-like receptors (TLRs), lectins (e.g., fibrinogen-related domain proteins - FReDs and C1q domain containing - C1qDC), thioester-containing proteins (TEPs), scavenger receptor cysteine-rich proteins (SRCRs), peptidoglycan recognition proteins (PGRPs) and gram-negative binding proteins (GNBPs) (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020).

After detection and recognition, signaling induces hemocytes to move to the reaction site and/or activate other humoral components (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). The main humoral effectors directly involved in pathogen killing are antimicrobial peptides (AMP). These molecules are categorized into defensins, big defensins, mytilins, myticins, and mytimacins (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). Other molecules that take part in humoral defense are bactericidal/permeability-increasing proteins (BPIs), proteases and lysozymes, heat shock proteins (HSPs), and protease inhibitors (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020).

As shown above, to compensate for the lack of an adaptative immune system, most marine invertebrates, including bivalves, resort to the production of a huge range of antimicrobial metabolites (Nalini et al., 2018). Many of these include proteins or peptides and can be investigated by proteomic approaches (Gerdol and Venier, 2015; Nalini et al., 2018; Sousa and Hinzmann, 2020). Through this, the protein profiles of organisms from different conditions can be compared to identify changes in protein expression, quantitative and qualitatively (Campos et al., 2012; Gonzalez and Pierron, 2015). Therefore, it is possible to understand organisms' responses at molecular levels as well as point out bioactive molecules of human interest (e.g., new compounds for the pharmaceutical industry) (Nalini et al., 2018; Sousa and Hinzmann, 2020). Additionally, proteins can be used as molecular biomarkers related to the biological effects of stressors, including bacteria exposure. Then, it can be useful for environmental monitoring, not to mention the asses of health status and/or food safety in commercial species (Campos et al., 2012; Gonzalez and Pierron, 2015).

The brown mussel *Perna perna* (Bivalvia, Mytilidae) is considered a valuable fishing resource for aquaculture, primarily in tropical and subtropical coastal regions (Cunha et al., 2014; Lourenço et al., 2012). This marine mussel is cultivated in countries such as Venezuela, South Africa, and primarily in Brazil (Calvo-ugarteburu et al., 2016; Narváez et al., 2008; Silva dos Santos et al., 2022a), that responds to 99.96% of *P. perna* global production (FAO, 2016). Because it is a sessile and filter-feeder, the mussel can accumulate pollutants from the water column and then provide a picture of its bioavailability (Birnstiel et al., 2019; Pusceddu et al., 2017; Silva dos Santos et al., 2022b). Therefore, *P. perna* has been proposed as a bioindicator species (Birnstiel et al., 2019; Pusceddu et al., 2017; Silva dos Santos et al., 2022b). Likewise,

the above-mentioned features make them possible vehicles for toxins, contaminants, and pathogenic bacteria for human beings (Neves et al., 2021; Silva dos Santos et al., 2022a). Since it is a pivotal commercial species, bacteria also can cause diseases and massive mortality on mussel farms, leading as well to relevant economic losses (Paillard et al., 2004).

Although international environmental rules instruct shellfish farming in places with good water quality (FAO, 2017), the mussel is still harvested and cultivated in contaminated areas exposed to bacteria (Silva dos Santos et al., 2022a, 2018). In this study, we aimed to assess the protein profile of the hepatopancreas of *P. perna* mussel challenged by bacteria to distinguish the molecular responses of introduced - *E. coli* and *S. enterica* - and marine indigenous bacteria - *V. parahaemolyticus*. The primary functions of the hepatopancreas are related to the metabolic balance, such as nutrient uptake and intracellular digestion, carbon and nitrogen metabolism, and detoxification (Rőszer, 2014). Nevertheless, it also acts directly and indirectly on immunity (Rőszer, 2014). Metabolic shifts occur induced by infection and impact the efficiency of the immune response (Rőszer, 2014). Directly, the organ is involved in antigen processing and pathogen clearance, besides being a source of several innate immunity molecules (Rőszer, 2014). As the bivalve hepatopancreas is a critical organ for innate immunity and metabolism, its protein profile furnishes a better understanding of the disturbance caused by bacteria and points to critical molecules in this process (Huang et al., 2021; Laith et al., 2021; Liu et al., 2014; Wu et al., 2013). To the authors' knowledge, this is the first study using the LC-MS/MS proteomic approach in *P. perna* then allowing to elucidate factors related to mussel-bacteria interaction. This knowledge can support the development of strategies and tools to be applied to coastal marine resource management and contribute to the sustainability of coastal systems, not to mention the finding of molecules of human interest.

2. Material and methods

2.1 Mussels sampling

Adult individuals of *P. perna* mussel (shell length: 5.3-8.2 cm) were manually collected on September 2021, from the rocky shores at Vermelha Beach (VB), Rio de Janeiro, Brazil (22° 57′ 18.59″ S and 43° 9′ 52.91″ W). Mussels and seawater were transported to the laboratory facilities using 20 L containers, prior washed with a 2% solution of neutral Extran (Merck, MA02) and distilled water. As shown in **Figure 1**, the beach is located at the mouth of Guanabara Bay, facing the Atlantic Ocean. According to Krepsky et al. (2020), VB composes the buffer zone of the Natural Monument of Sugar Loaf and Urca Hill’s conservation unit and presents excellent water quality by microbiological standards.

Fig 1. Vermelha Beach (VB), Rio de Janeiro, Brazil: mussels and seawater sampling location.

2.2 Bacterial suspensions preparation

Escherichia coli 2348/EPEC and *S. enterica* subsp. *enterica* serovar Typhimurium str. SL1344 were grown at Tryptic Soy Broth (TSB; Himedia, Brazil) at

37 °C for 20 h, still in the bacteria exponential growth phase. Under the same conditions, *V. parahaemolyticus* ATCC 17802 was incubated in Alkaline Peptone Water 1% (1% peptone and 1% NaCl). Bacteria were washed twice by centrifuging at 3000 g and 15 °C for 10 min, and resuspended into PBS-NaCl (pH 7.4; Parisi et al., 2008). Bacteria suspension concentrations were obtained using the standard 0.5 (1.5 x 10^8 CFU mL^{-1}) of the McFarland Scale and further diluted into sterile PBS-NaCl to 1.5 x 10^7 CFU mL^{-1}.

The above-mentioned strains were obtained from the Laboratory of Enteropathogens, Veterinary, Environmental and Food Microbiology/Department of Microbiology and Parasitology/Biomedical Institute/Fluminense Federal University – UFF; Laboratory of Pathogenic cocci and Microbiota/Department of Medical Microbiology/Professor Paulo de Goés Institute of Microbiology/Federal University of Rio de Janeiro – UFRJ; and Enterobacteria Laboratory/Oswaldo Cruz Institute/Oswaldo Cruz Foundation - FIOCRUZ, respectively.

2.3 **Experimental design and challenge**

At the laboratory, mussel shells (**Figure 2**) were cleaned to remove encrusting organisms and randomly distributed into 5 groups (n = 9) representing the following conditions: Non-injected control (NC); Injected control (IC); *Escherichia coli* (EC); *Salmonella enterica* (SE); and *Vibrio parahaemolyticus* (VP). Mussels from the bacteria-challenging groups were carefully injected with 100 µL of 1.5 x 10^7 CFU mL^{-1} of the corresponding bacterial suspension on their posterior adductor muscle (**Figure 2**), using a sterile syringe. Non-injected (NC) and injected (IC) control groups comprised, respectively, mussels not challenged and mussels injected with 100 µL sterile PBS-NaCl. After injections, mussels (n=9) were placed into separated glass aquaria, previously cleaned with neutral Extran solution (2% in distilled water), according to each condition. Aquariums were filled with 3 L of filtered seawater from VB (20 µm; salinity 30.9) and kept with constant aeration at 18 °C. Mussels were dissected 24h post-incubations using sterile stainless-steel scalpel and forceps, their hepatopancreas (**Figure 2**) were removed and individually conditioned in sterile Eppendorf tubes (2 mL), flash-frozen in liquid nitrogen, and kept in a freezer (-20°C) until lyophilization.

Fig 2. The external and internal anatomical features of the mussel *Perna perna*.

2.4 Total protein extraction and digestion

Lyophilized samples were added to microtubes containing 500 µL of extraction buffer (2M thiourea, 7M urea, 4% CHAPS, 50 mM tris-HCl; pH = 8.5), 5 µL of protease inhibitor and ceramic beads (mix of 1.4 mm and 2.8 mm). Samples were mechanically homogenized using FastPrep – 24™ 5G (MP Biomedicals) in the following mode: speed = 6.5 m/s; adapter = coolprep; time = 40 s. The program was run 3 times at the above conditions, with intervals of 30 s between each running and adding dry ice. Since the samples were not liquid enough, the above process was repeated by adding the same above amount of extraction buffer and protease inhibitor. Then, an equal volume of trichloroacetic acid/acetone (20/80) solution was added to the samples,

which precipitated at 4 °C for 45 min. Samples were then centrifuged at 20.000 g and 4 °C for 50 min, and washed 5 times with acetone/tris-HCl 6.8 100mM (70/30) solution added by bromophenol blue pH indicator. Thereafter, samples were redissolved in the extraction buffer (500 µL) added to 1% of protease inhibitor, and diluted 100 times for protein quantification by the Bradford method (Bradford, 1976). Based on protein concentration, samples from each experimental condition were chosen (IC, EC, and SE: n = 5 and NC and VP: n = 4) to proceed to the next steps.

The volume necessary for 10 µg of total proteins was transferred to clean tubes and then samples were reduced with 6 µL dithiothreitol (final concentration 50 mM) for 20 min at room temperature. Then, 14 µL of acrylamide 30% were added to the samples (final concentration 20%). After shaking for 20 min, 5µL of tetramethylethylenediamine (TEMED) and 5 µL of ammonium peroxydisulfate 10% were added to each tube for gel polymerizing. Thereafter, samples were fixed with 100 µL of methanol, acetic acid, and H_2O (50/40/10) solution for 10 min. About 500 µL of urea (6 M) were added to each tube and, after 10 min of shaking, 250 µL of acetonitrile were joined, vortexed and removed. Samples were rehydrated in 200 µL of ammonium bicarbonate (100 mM) and incubated for 10 min under shaking. An equal volume of acetonitrile (200 µL) was added, vortexed and removed. Once again, 200 µL of acetonitrile was added, and after complete sample dehydration, the liquid was removed and samples were left to air-dry. Five µg of each protein extract were prepared using a modified Gel-aided Sample Preparation protocol (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4409837/). Samples were digested with trypsin/Lys-C overnight at 37 °C. For nano-LC fragmentation, protein or peptide samples were first desalted and concentrated onto a µC18 Omix (Agilent) before analysis.

2.5 LC-MS/MS analysis

The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra-high-pressure nano flow chromatography system. Approximatively 200 ng of each peptide sample were concentrated onto a C18 pepmap 100 (5 mm x 300 µm i.d.) precolumn (Thermo Scientific) and separated at 50 °C onto a reversed phase Reprosil column (25cm x 75µm i.d.) packed with 1.6 µm C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1 % formic acid in 99.9 % ACN (v/v) (B). The nanoflow rate was set at 250 nl/min, and the gradient profile was as follows: from 2 to 30 % B within 70 min, followed by an increase to 37 % B within 5 min and further to 85 % within 5 min and reequilibration.

MS experiments were carried out on an TIMS-TOF pro mass spectrometer (Bruker Daltonics) with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). A 1400 spray voltage with a capillary temperature of 180°C was typically employed for ionizing. MS spectra were acquired in the positive mode in the mass range from 100 to 1700 m/z and 0.60 to 1.60 1/k0 window. In the experiments described here, the mass spectrometer was operated in PASEF DIA mode with exclusion of single charged peptides. The DIA acquisition scheme consisted of 16 variable windows ranging from 400 to 1200 m/z.

2.6 **Protein identification**

Database searching and LFQ quantification (using XIC) was performed using DIA-NN (version 1.8; https://www.nature.com/articles/s41592-019-0638-x). An updated UniProt *Mytilus coruscus* database was used for library-free search/library generation. In the absence of genomic data for the species *Perna perna* or within the genus Perna, *Mytilus coruscus* was the closest species from *Perna perna* (same sub-family, Mytilinae) with the most comprehensive available proteome (Li et al., 2020). The default parameter 0.0 was used for RT prediction and extraction mass accuracy, which means DIA-NN performed automatic mass and RT correction. Top six fragments (ranked by their library intensities) were used for peptide identification and quantification. The FDR was set to 1% at the peptide precursor level. The variable modifications allowed were as follows: Nterm-acetylation and Oxidation (M). In addition, C-Propionoamide was set as fix modification. “Trypsin/P” was selected. Data were filtering according to a FDR of 1%. Cross-run normalisation was performed using RT-dependent.

2.7 Data analysis

For the statistical analyses, label-free quantitative data were imported into the software Perseus 1.6.15.0 (Tyanova et al., 2016). Protein intensity values were $\log_2$-transformed and only identified proteins in a minimum of three samples of at least one of the tested conditions (i.e., non-injected control - NC; injected control - IC; *Escherichia coli* - EC; *Salmonella enterica* - SE; and *Vibrio parahaemolitycus* - VP) followed to the further analysis. Missed values were replaced from the normal distribution (width = 0.3 and downshift = 1.8).

Data from the different conditions were compared based on a one-way ANOVA (Benjamini-Hochberg FDR; significance ≥ 0.05) and *a posteriori* Tukey HSD (significance ≥ 0.05) tests. Protein functional annotation was carried out using the tools UniProt (https://www.uniprot.org/) and EggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale (http://eggnog-mapper.embl.de/; Cantalapiedra et al., 2021). Then, the biological role of significantly different proteins fronting bacterial challenge was discussed taking into account the Clusters of Orthologous Group (COG) categories, biological process Gene Ontology (GO) terms, and literature review.

The UpSet graph for visualizing the intersecting sets of significantly different proteins among the tested conditions was created using the UpSetR package available at https://upset.app/ (Conway et al., 2017; Lex, 2014).

3. Results and Discussion

A total of 3,805 proteins were found in the hepatopancreas of *Perna perna* mussels that underwent five different conditions (non-injected control - NC; injected control - IC; *Escherichia coli* - EC; *Salmonella enterica* - SE; and *Vibrio*

parahaemolyticus - VP), of which 378 are uncharacterized (available as supplementary data). From the total, 597 were significantly different among conditions – upregulated or downregulated in at least one condition. **Figure 3** shows the number of proteins significantly different for each intersecting group from pairwise comparisons. Eleven proteins showed significant differences between the two control groups (available as supplementary data), probably in response to the stress related to the injection, and for this reason were not discussed here. Mussels injected with *V. parahaemolyticus* presented a more marked response, with 343 proteins significantly different from all the other conditions that are discussed in an independent topic (3.2). Among the remaining significantly different proteins, only that different from both non-injected (NC) and injected (IC) controls are discussed herein (Topic 3.1).

Fig 3. Upset plot for visualizing intersecting sets of significantly different proteins among the tested conditions. NC - non-injected control; IC - injected control; EC - *Escherichia coli*; SE - *Salmonella enterica*; and VP - *Vibrio parahaemolyticus*. SE_EC means a significant difference between these conditions and so on.

3.1 Response to bacterial challenge

Table 1 shows the 31 proteins that were upregulated or downregulated for one or more bacterial challenge groups (*Escherichia coli* - EC; *Salmonella enterica* - SE; and *Vibrio parahaemolyticus* - VP) compared with both controls (non-injected - NC and injected - IC). These proteins are discussed below according to each challenge group of *P. perna*.

3.1.1 *Escherichia coli*

Mussels challenged with *E. coli* presented only two proteins downregulated compared to controls, namely: a von Willebrand Factor A (vWA) domain-containing protein and a FERM (“four-point-one, ezrin, radixin, moesin”) domain-containing protein. According to Whittaker and Hynes (2002), the most ancient vWA domain-containing proteins settle the intracellular environment of all eukaryotes and take part in transcription, DNA repair, ribosomal and membrane transport, and the proteasome. Probably, vWA domains mediate protein-protein interactions of multiprotein complexes involved in these functions (Whittaker and Hynes, 2002). Afterward in the evolution, vWA domains emerged in extracellular proteins of metazoan. Among these, the most representative in the extracellular matrix are the integrin β subunits, the major cell surface receptors, and also involved in cell adhesion (Whittaker and Hynes, 2002). Finally, nematodes and chordates separately diversified extracellular matrix proteins containing vWA domains, which compose the majority of known vWA domain-containing proteins (Whittaker and Hynes, 2002). In general, the vWA domains are involved in protein-protein interactions, frequently using divalent cations such as Ca^{+2} (Bowden et al., 2020; Whittaker and Hynes, 2002). Some mollusks' studies showed the vWA domain's important role in defense and immunity. Smith et al., (2017) found matrilin-like proteins rich in the vWA domain in the glue of the slug *Arion subfuscus*.

These proteins jointly with lectin and C1q domains seem to have a defensive role in the slug glue, one of the first barriers against pathogens. Bowden et al. (2020) showed post-translational regulation of relevant pathways for immunity and metabolism via deimination of several proteins found in the hemolymph of the eastern oyster *Crassostrea virginica*, including vWA domain-containing protein.

The second protein downregulated in *P. perna* mussels injected with *E. coli*, compared to the controls, was a FERM domain-containing protein. A protein like that was also found in the transcriptome analysis of the marine gastropod *Concholepas concholepas* (Cárdenas et al., 2011). The superfamily of proteins that hold a FERM domain is ubiquitous and performs the linkage between the plasm membrane and actin cytoskeleton, sometimes mediating the activation of their binders (Frame et al., 2010; Tanentzapf and Brown, 2006; Tepass, 2009). The FERM domain can interact with membrane lipids and several protein-binding partners, including transmembrane ion channels, adhesion molecules (e.g., integrins), and cytoplasmic proteins. Therefore, these proteins act in the structure, transport, and signal transduction of cells (Frame et al., 2010; Tanentzapf and Brown, 2006; Tepass, 2009).

Figure 4 graphically sums up the immune-related role of these two down regulated proteins after *E. coli* challenging in a ciliated hepatopancreatic cell of *P. perna* mussel. The von Willebrand Factor A domain-containing protein (vWA) seems to behave as a humoral effector in mollusks, primarily in the extracellular matrix. The FERM domain-containing protein (FERM), in turn, is probably located close to the plasma membrane and involved in signal transduction, therefore acting at the initial phases after pathogen recognition to trigger some immune response.

Fig 4. Immune-related role of the altered proteins in a ciliated hepatopancreatic cell of *Perna perna* mussel, 24h post-*Escherichia coli* injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). In mussels challenged with *E. coli*, a von Willebrand Factor A domain-containing protein (vWA) and a FERM domain-containing protein (FERM) were downregulated. FERM is probably located close to the plasma membrane and takes part in signal transduction. VWA seems to act as a humoral effector in mollusks, primarily in the extracellular matrix.

3.1.2 *Salmonella enterica*

Similar to mussels injected with *E. coli*, the FERM domain-containing protein was downregulated in mussels challenged by *S. enterica*. Likewise, the proteins splicing factor 3A subunit 1 (SF3A1), actin beta/gamma 1 (ACTB_G1), and galactokinase 2 (GALK2) were deregulated after *S. enterica* injection, whereas the following proteins were upregulated: leucine-rich repeat and guanylate kinase domain-containing protein (LRGUK), stress-activated protein kinase JNK, 60S ribosomal protein L18a (RPL18A), proteasome activator PA28γ (PA28γ or PSME3), Mitochondrial fission process protein 1 (MTFP1), Tubulin beta chain (β-Tubulin), axonemal dyneins (DNAH3 and DNAH9),

EFHC1, Cilia- and flagella-associated protein 299, Glycerol-3-phosphate dehydrogenase (GPD2), Oxoglutarate dehydrogenase (OGDHL).

The protein LRGUK presents two essential domains for humoral immune response in bivalves - leucine-rich repeat (LRR) and guanylate kinase (GK). The first one characterizes a toll-like receptor (TLR), in other words, a membrane-bound pattern recognition receptor (PRR) (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). These receptors are crucial to detect countless classes of PAMPs (pathogen-associated molecular patterns) at microbial surfaces (e.g., LPS, components of the bacterial cell wall, flagellin, among others) and, therefore, trigger the immune response (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). According to Gerdol and Venier (2015), TLRs consist of an evolutionarily conserved structure as follows: an intracellular toll-interleukin-1-receptor domain; a transmembrane region; and a variable extracellular region with an LRR domain. In the case of LRGUK found upregulated in the *P. perna* hepatopancreas, the intracellular domain seems to be a guanylate kinase (GK).

Membrane-associated guanylate kinases (MAGUKs) are a conserved protein family involved with tissue development, cell-cell communication, cell adhesion, and signal transduction (Olsen and Bredt, 2003; Zhu et al., 2012, 2011). Originally, GKs contain a GMP-binding site that catalyzes GMP to GDP conversion. However, several studies have shown that MAGUKS evolved, currently acting on protein-protein interaction (Olsen and Bredt, 2003; Zhu et al., 2012, 2011). Accordingly, the LRGUK protein found in this study seems to hold an LRR domain for pathogens recognition, and a GK domain performing signal transduction (e.g., interacting with microtubule-associated protein; Reese et al., 2007). Likewise, Huang et al. (2018) found a new transmembrane LRR (leucine-rich repeat and fibronectin type-III domain-containing protein) in the pacific oyster *Crassostrea gigas*. It was observed that the protein acts as a pattern recognition receptor for *Vibrio* spp. and promotes hemocyte phagocytosis. Additionally, it was found widely expressed in several tissues such as gills, adductor muscles, digestive glands, and hemocytes (Huang et al., 2018).

Stress-activated protein kinase JNK participates in the mitogen-activated protein kinase (MAPK) cascade (Hatanaka et al., 2009; Irazoqui et al., 2010; Sun et al., 2016). MAPK cascade is a signaling pathway associated with innate immunity, evolutionarily conserved from invertebrates to mammals (Hatanaka et al., 2009; Irazoqui et al., 2010; Sun et al., 2016). Excluding variations, in invertebrates the pathway consists of three categories of protein kinases that are sequentially phosphorylated, activating the following ones (Hatanaka et al., 2009). In *Drosophila* and mammals, JNK or p38 protein kinases phosphorylate transcription factors at the end of the cascade, regulating the transcriptional activity of host response genes (Hatanaka et al., 2009). In the Yesso scallop *Patinopecten yessoensis*, it seems that only the JNK kinase participates in the immune response against Gram-negative and -positive bacteria (Sun et al., 2016). In this way, after pathogen recognition by TLRs, the cascade is triggered and results in the transcription of host response genes that encode proteins related to innate immune response (e.g., antimicrobial peptides) (Hatanaka et al., 2009; Irazoqui et al., 2010; Sun et al., 2016).

After recognition and signaling, the transcription of response genes and mRNA processing occurs in the nucleus. The protein splicing factor 3A (SF3A) complex accomplishes mRNA splicing via spliceosome and is composed of three proteins, as follows: SF3A1, SF3A2, and SF3A3 (De Arras and Alper, 2013). Hence, different mRNAs are constructed from the pre-mRNA splicing, depending on the cell demands. However, all three SF3A subunits (SF3A1, SF3A2, and SF3A3) are required for mRNA splicing (De Arras and Alper, 2013). In this study, the subunit SF3A1 was downregulated after *S. enterica* injection, suggesting a splicing brake by SF3A to build an alternative mRNA. Indeed, De Arras and Alper (2013) found the SF3A complex as a regulator of the innate immune response in mouse macrophages. If splicing by SF3A occurs or not, two alternative mRNA are created and drive to opposed immune responses (De Arras and Alper, 2013). The processed mRNA moves for translation by ribosomes at the cytoplasm. Herein, the 60S ribosomal protein L18a (RPL18A) was upregulated in response to *S. enterica* challenge. RPL18A is a structural constituent of ribosomes and its upregulation suggests an increase in protein translation (Aoyama et al., 1989).

Proteasome activator PA28γ (PA28γ or PSME3) was also upregulated in *P. perna* in response to *S. enterica* challenge. Proteasomes are barrel-shaped protease complexes that perform the majority of the proteolytic processes in eukaryotic cells (Fort et al., 2015; Rechsteiner and Hill, 2005). Therefore, proteasomes are involved in countless basic cellular functions, such as protein quality control and cellular regulation (Fort et al., 2015; Rechsteiner and Hill, 2005). However, the binding of proteasome activators (PAs) is required to make proteasomes enzymatically active. The proteasome activator PA28 presents three homologs, namely: PA28α, PA28β, and PA28γ (Fort et al., 2015; Rechsteiner and Hill, 2005). The two first mentioned are restricted to jawed vertebrates and are related to important immune functions. On the other hand, PA28γ is found starting from the early invertebrates and is likely involved in transcription or apoptosis since it occurs in the nucleus (Fort et al., 2015; Rechsteiner and Hill, 2005).

Another protein upregulated in *P. perna* mussels was MTFP1, which acts as a receptor to mediate mitochondrial fission in yeast and mammalian cells (Sheridan and Martin, 2010; van der Bliek et al., 2013). In healthy cells, mitochondria continually fuse/divide and move through the cytoskeletal in response to cellular processes and to maintain the balance of the mitochondrial network (Sheridan and Martin, 2010; van der Bliek et al., 2013). However, according to van der Bliek et al. (2013), fusion decreases and/or fission increases under cellular stress. Additionally, fission considerably rises during apoptosis (Sheridan and Martin, 2010; van der Bliek et al., 2013). Therefore, MPFP1 upregulated in *P. perna* hepatopancreas suggests increased mitochondrial fission and, possibly, apoptosis. Indeed, *S. enterica* is a facultative intracellular pathogen that can survive and proliferate in the intracellular environment (Jantsch et al., 2011; Kehl et al., 2020). Apoptosis may be part of mussel cells' defense against *S. enterica*, to remove damaged and infected cells.

Several proteins related to cytoskeleton structure and cell motility significantly responded to Salmonella injection. ACTB_G1 was downregulated, whereas β-tubulin, axonemal dyneins (DNAH3 and DNAH9), EFHC1, and cilia- and flagella-associated protein 299 were upregulated in *P. perna* hepatopancreas compared with controls. Actin

monomers (ACTB_G1) polymerize into actin filaments that, in turn, are arranged into bundles or networks at the cell edge (Fletcher and Mullins, 2010; Ramaekers and Bosman, 2004). Actin filaments are usually associated with the membrane proteins and are continually polymerized and depolymerized in response to local signaling (Fletcher and Mullins, 2010; Ramaekers and Bosman, 2004). As a result, actin drives cell movement and shape (e.g., pseudopods and phagocytosis) and cell-cell communication (Fletcher and Mullins, 2010; Ramaekers and Bosman, 2004). β- and α-tubulin heterodimers compose microtubules that handle the anchoring and transport of vesicles and organelles (Fletcher and Mullins, 2010; Ramaekers and Bosman, 2004).

Microtubules, together with axonemal dyneins, also constitute cilia and flagella (Mirvis et al., 2018; Smith et al., 2020). Dyneins are microtubule-associated motors that hydrolyze ATP to mediate cilia and flagella movement (Mirvis et al., 2018; Smith et al., 2020). EFHC1 is a microtubule-associated protein in humans, conserved in cells that hold cilia and/or flagella. The protein holds an EF-hand motif at the C-terminus that binds Ca^{+2} (Ikeda et al., 2005; Murai et al., 2008; Rossetto et al., 2011). Cilia-and flagella-associated proteins act on the assembly of protein complexes, such as axoneme (Feng et al., 2022). Indeed, hepatopancreatic tubules of bivalves comprise two types of cells, as follows: cylindrical-shaped acidophilic digestive cells and pyramid-shaped basophilic cells (Tunali and Erkan, 2008; Yang et al., 2021). In most bivalve species, basophilic cells have cilia on their apical surfaces and are specialized in protein secretion (Tunali and Erkan, 2008; Yang et al., 2021). According to Mirvis et al. (2018) and Smith et al., (2020), actin can promote cilia disassembly. Therefore, the above protein pattern with actin downregulated and all the other ciliary proteins upregulated suggests an increased cilia activity, probably related to the secretory role of these cells.

The enzymes GALK2, GPD2, and OGDHL act on energy production and metabolism (Nelson and Cox, 2017). GALK2 converts D-galactose into α-D-galactose-1-phosphate, the first reaction to form glucose-6-phosphate from galactose (Nelson and Cox, 2017). Afterward, glucose-6-phosphate can proceed to glycolysis or glucogenesis pathways (Nelson and Cox, 2017). GPD2 catalyzes the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate, an intermediate in the glycolytic pathway (Nelson and Cox, 2017). It is worth highlighting that glycerol is a lipid constituent, converted into glycerol-3-phosphate by the enzyme glycerol kinase, to be metabolized in glycolysis (Nelson and Cox, 2017). Moreover, GPD2 participates indirectly in the respiratory chain (Nelson and Cox, 2017). Last but not least, OGDHL converts 2-oxoglutarate into S-succinyl dihydrolipoamide in the tricarboxylic acid (TCA) cycle (Nelson and Cox, 2017). The enzyme is a major determinant of the metabolic flux in the TCA cycle since 2-oxoglutarate is a key intermediate in carbon metabolism in that cycle and also acts as the major carbon skeleton for nitrogen-assimilatory reactions (Huergo and Dixon, 2015; Nelson and Cox, 2017). Therefore, the enzyme fluctuates depending on carbon and nitrogen availability and energy demand (Huergo and Dixon, 2015; Nelson and Cox, 2017).

The previously mentioned scenario suggests high energy consumption and metabolism regulation as a result of the *S. enterica* challenge. Indeed, cellular and molecular immune responses demand a high energy cost (Brokordt et al., 2019). According to Sokolova et al. (2012), energy balance is crucial for aquatic invertebrates

to cope with stress. During moderate stress, the energy demand to cover protection and damage repair increases (Sokolova et al., 2012). GALK2 downregulated and GPD2 upregulated in *P. perna* hepatopancreas suggest a change from carbohydrate to lipid metabolism, together with OGDHL upregulated to maintain the raised flux in the TCA cycle and the energy production. Similarly, a significant decline in glycogen reserves was observed in the hepatopancreas of the scallop *Chlamys farreri*, 3 h after *Vibrio anguillarum* challenge (Wang et al., 2012). These results corroborate and show the preference of bivalves to use carbohydrate reserves as a fast energy source for immune response. Under normal circumstances and moderate stress, aerobic metabolism is enough to overlay energy demand and is mostly fueled by lipids and carbohydrates (Sokolova et al., 2012). Proteins are metabolized only during extreme energy deficiency (Sokolova et al., 2012).

An overview of altered proteins - downregulated or upregulated - in the hepatopancreas of *P. perna* mussel in response to the *S. enterica* injection is shown in **Figure 5**. Before all else, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) acts on pathogens recognition - through the LRR domain which characterizes a membrane-bound toll-like receptor (TLR) - and signal transduction - via the GK domain. As shown by the mussels injected with *E. coli*, the FERM domain-containing protein (FERM) possibly takes part in signal transduction close to the plasma membrane. At the end of the mitogen-activated protein kinase (MAPK) cascade, the stress-activated protein kinase JNK (JNK) phosphorylates transcription factors that, in turn, regulate the transcription of response genes. The proteasome activator PA28γ (PA28γ) located in the nucleus is also presumably involved in transcription or apoptosis. After transcription, the splicing factor 3A subunit 1 (SF3A1) takes part in mRNA processing, which is translated at the cytoplasm by ribosomes constituted by the 60S ribosomal protein L18a (RPL18A). Mitochondrial fission process protein 1 (MTFP1) mediates mitochondrial fission, a process that rises under cellular stress and during apoptosis. The proteins actin beta/gamma 1 (ACTB_G1), Tubulin beta chain (β-Tubulin), axonemal dyneins (DNAH3 and DNAH9), EFHC1, Cilia- and flagella-associated protein 299 (CF-299) constitute cytoskeleton and cilia structure, which are probably related to the protein secretion function of the ciliated basophilic cells of hepatopancreas. Last but not least, the enzymes GALK2, GPD2, and OGDHL (not shown in **Figure 5**) act on energy production and metabolism, suggesting a high energy consumption and metabolism regulation to deal with *S. enterica* challenge.

Fig 5. Immune-related role of the altered proteins - downregulated or upregulated - in a ciliated hepatopancreatic cell of *Perna perna* mussel, 24 h post-*Salmonella enterica* injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). First of all, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) acts on pathogens recognition and signal transduction, through the LRR and GK domains, respectively. As in the mussels injected with *E. coli*, the FERM domain-containing protein (FERM) probably also takes part in signal transduction close to the plasma membrane. Afterward, the stress-activated protein kinase JNK (JNK) participates at the end of the mitogen-activated protein

kinase (MAPK) cascade, phosphorylating transcription factors that regulate the transcription of response genes. The proteasome activator PA28γ (PA28γ) located in the nucleus is also presumably involved in transcription or apoptosis. After transcription, the splicing factor 3A subunit 1 (SF3A1) takes part in mRNA processing, which is translated at the cytoplasm by ribosomes constituted by the 60S ribosomal protein L18a (RPL18A). Mitochondrial fission process protein 1 (MTFP1) mediates mitochondrial fission, which rises under cellular stress and during apoptosis. Last but not least, the proteins actin beta/gamma 1 (ACTB_G1), Tubulin beta chain (β-Tubulin), axonemal dyneins (DNAH3 and DNAH9), EFHC1, Cilia- and flagella-associated protein 299 (CF-299) constitute cytoskeleton and cilia structure, which are probably related to the protein secretion function of the ciliated basophilic cells of hepatopancreas.

3.1.3 *Vibrio parahaemolyticus*

Regarding mussels' proteomic response to *V. parahaemolyticus*, the proteins leucine-rich repeat and guanylate kinase domain-containing protein (LRGUK), splicing factor 3A subunit 1 (SF3A1), 60S ribosomal protein L18a (RPL18A), β-Tubulin and EFHC1 presented the same profile than mussels challenged with *S. enterica*. That is, the protein SF3A1 was downregulated and all the others were upregulated compared with controls. Hence, there were similar response patterns primarily concerning recognition, signaling, transcription, and translation, besides cytoskeleton and cilia-associated proteins. Additionally, for mussels injected with *V. parahaemolyticus*, the proteins rhomboid protease (RHBDL1_2_3), RNA 3-phosphate cyclase-like 1 (RCL1), and Histone H2A were downregulated, whereas the following proteins were upregulated: hepatocyte nuclear factor-4α (HNF4α or NR2A1), poly-U-binding factor 60 kDa (PUF60), THOC1, nuclear GTP-binding protein (NUG1), ABC transmembrane type-1 domain-containing protein, motor dyneins (DNAH), ARMC3, malate dehydrogenase (MDH2) and mitochondrial-processing peptidase subunit beta (PMPCB).

A rhomboid protease (RHBDL1_2_3) was downregulated in *P. perna* hepatopancreas in response to Vibrio challenge. Rhomboid proteases compose a subfamily of enzymes that catalyze intramembrane proteolysis (Urban and Dickey, 2011). These serine endopeptidases are found in all domains of life and, in animals, act by initiating cell signaling (Urban and Dickey, 2011). For example, in *D. melanogaster* and *C. elegans*, rhomboid proteases mediate the activation of epidermal growth factor receptor (EGFR) signaling to trigger several pathways related to development processes (Dutt et al., 2004; Van Buskirk and Sternberg, 2007; Wasserman et al., 2000; Yogev et al., 2008). One of the main pathways activated by EGFR is the MAPK cascade (Guichard et al., 2000; Moghal and Sternberg, 2003), discussed in the prior section (3.1.2). MAPK pathway regulates the cell's transcriptional activity, including that related to immune responses (Hatanaka et al., 2009). Therefore, rhomboid proteases may have some role in signal transduction involved in defense against bacteria in *P. perna*, probably after recognition and signaling by leucine-rich repeat and guanylate kinase domain-containing protein (LRGUK) (discussed in the prior section 3.1.2).

Another protein upregulated in Vibrio-challenged mussels was the hepatocyte nuclear factor-4α (HNF4α or NR2A1). NR2A1 is part of a superfamily of transcription factors called nuclear receptors (NRs) (Miglioli et al., 2021; Sladek, 2011). Through two highly conserved domains - DNA binding (DBD) and ligand binding (LBD)

domains - NRs regulate transcription in response to small lipophilic ligands (Miglioli et al., 2021; Sladek, 2011). Hence, NR2A1 controls several hepatic functions at the transcriptional level (Jiang et al., 2021). In mammals, NR2A1 regulates several genes related to hepatocyte differentiation and the control of lipids, lipoproteins, and glucose metabolism, among other functions (Jiang et al., 2021). In Drosophila, Storelli et al. (2019) showed NR2A1 as a crucial regulator of lipid metabolism in early adulthood. HNF4α drives the production of very long-chain fatty acids (VLCFA) and VLCFA-derived hydrocarbons from lipid stores (Storelli et al., 2019). However, these lipids are not fated for energy production but are secreted and transported to the body surfaces to act as waterproofing agents and pheromones (Storelli et al., 2019). Through this, HNF4 also maintains glucose homeostasis (Storelli et al., 2019). A similar process also occurs in mice, whereby HNF4a from hepatocytes acts on the expression of fatty acids elongases that contributes to the waterproof epidermis (Storelli et al., 2019). Therefore, NR2A1 upregulated in *P. perna* hepatopancreas indicates the regulation of hepatic genes likely related to lipid metabolism, not necessarily for energy production but for some process to deal with the bacterial challenge.

After transcription, mRNA is processed to form messenger ribonucleoprotein complexes (mRNPs) to be translated in the cytoplasm (Kew et al., 2020; Li and Guan, 2021; Ren et al., 2015). Splicing is an essential step in mRNA processing, whereby protein-coding sequences known as exons are attached together after removing non-coding sequences (introns) from the primary transcripts (Kew et al., 2020; Li and Guan, 2021; Ren et al., 2015). Hence, different combinations of exons are possible to form alternative mRNAs, which allows expanding the range of proteins encoded from the same primary sequence (Kew et al., 2020; Li and Guan, 2021; Ren et al., 2015). Splicing is catalyzed by a family of specialized proteins called splicing factors, including poly-U-binding factor 60 kDa (PUF60). PUF60 seems to have a direct role in the modulation of innate immunity (Kew et al., 2020; Li and Guan, 2021; Ren et al., 2015). When the sea cucumber *Stichopus monotuberculatus* was challenged with lipopolysaccharides (LPS) - a constituent of the extern membrane of Gram-negative bacteria assumed as a PAMP - PUF60 mRNA was upregulated (Ren et al., 2015). Additionally, PUF60 overexpressed may induce apoptosis in the sea cucumber (Ren et al., 2015). In the Japanese flounder *Paralichthys olivaceus*, PUF60 gene expression was upregulated after bacterial infection and, in contrast, was downregulated after viral infection (Li and Guan, 2021). Additionally, after PUF60 knockdown, bacterial dissemination in the tissues of the flounder rose whereas viral replication was blocked (Li and Guan, 2021). In the opposite situation, PUF60 overexpression inhibited bacterial dissemination but promoted viral replication (Li and Guan, 2021). Similar to the sea cucumber, PUF60 was found to be involved in autophagy and apoptosis processes in the Japanese flounder (Li and Guan, 2021; Ren et al., 2015). In *C. elegans*, bacterial infection induces a PUF60-dependent splicing remodeling (Kew et al., 2020). Kew et al. (2020) showed that PUF60 leads to sensitivity to bacterial infection, but increases lifespan likely by limiting the inflammatory process induced by infection. Therefore, PUF60 upregulated in *P. perna* challenged by Vibrio corroborates with the above-mentioned results and suggests its role in immunity modulation in response to bacterial infection.

Still about mRNA processing, the protein THOC1 is part of the multi-subunit THO complex that, in turn, composes the transcription and export (TREX) complex (Jimeno and Aguilera, 2010; Reed, 2003). TREX complex is found in all eukaryotes and also takes part in mRNA processing to be transported from the nucleus to the cytoplasm as messenger ribonucleoprotein complexes (mRNPs) (Jimeno and Aguilera, 2010; Reed, 2003). Particularly, the THO complex couple transcription and export, as it has a functional role related to RNA polymerase II (RNAPII) transcription elongation and mediates mRNA export (Castellano-Pozo et al., 2012; Jimeno et al., 2002; Rehwinkel et al., 2004). Additionally, it was shown that the THO complex has a key role in the cell cycle and differentiation (Castellano-Pozo et al., 2012). Therefore, THOC1 (homolog Hpr1 in Drosophila, *C. elegans*, and yeast) upregulated in *P. perna*, together with other proteins, suggests an increase in protein transcription and translation activity in the cell in response to a signal. Indeed, the Vibrio-challenged group had the highest response, with approximately 500 proteins upregulated or downregulated. Thus, it seems that THOC1 and, therefore, THO complex, have some role in the transcription and translation of proteins related to the immune response in the mussel *P. perna*.

Two proteins related to ribosome biogenesis were altered in response to Vibrio challenge - RCL1 downregulated and NUG1 upregulated. The primary transcripts of rRNAs for the small and large ribosomal subunits are co-transcribed as a single transcript to be cleavage afterward (Horn et al., 2011; Zhu et al., 2021). RCL1 is an evolutionarily conserved endonuclease that mediates this cleave (Horn et al., 2011; Zhu et al., 2021). In yeasts, RCL1 cleaves the pre-rRNA at the A2 site (Horn et al., 2011), whereas in zebrafish cleaves at the A1 site (Zhu et al., 2021). Therefore, depletion in RCL1 impairs ribosome maturation (Horn et al., 2011; Zhu et al., 2021), and at the phenotypical level in zebrafish, rcl1-/- mutants exhibited alterations in the digestive organogenesis, such as a diminutive liver (Zhu et al., 2021). According to Zhu et al. (2021), RCL1 deficiency possibly triggers an ordinary mechanism that upregulates the expression of other genes related to ribosome biogenesis. Indeed, concomitant to RCL1 downregulation, GTPase NUG1 (nucleostemin in humans) was upregulated.

NUG1 is essential for 60S ribosome (large subunit) assembly and nuclear export (Bassler et al., 2006; Manikas et al., 2016). The enzyme consists of a GTPase middle domain flanked by the N- and C-terminal domains (Bassler et al., 2006; Manikas et al., 2016). According to Bassler et al. (2006), the N-terminal domain is crucial for nucleolar targeting and association with pre-60S particles since it displays RNA binding activity. The highly evolutionary-conserved C-domain is also essential for ribosome biogenesis, although its function is not well known (Bassler et al., 2006; Manikas et al., 2016). The central GTPase domain is stimulated by potassium ions and, as opposed to the other domains, plays a non-essential regulative role in pre-60 S subunit biogenesis (Manikas et al., 2016). Manikas et al. (2016) showed that NUG1 and the RNA helicase DBP10 bind close to each other in the pre-ribosome, where posteriorly the peptidyl-transferase center (PTC) is localized in the mature ribosome. Therefore, it suggests these enzymes are involved in the formation of the PTC in ribosomes. Defects or depletion of NUG1 results in ribosome 60S biogenesis issues (Manikas et al., 2016). In *C. elegans*, NUG1 has a role in cell growth and proliferation by enabling ribosome biogenesis (Kudron and Reinke, 2008). In Drosophila, NUG1 depletion impairs midgut precursor cells and cell

growth likely as a result of blocking ribosome biogenesis (Rosby et al., 2009). Therefore, it seems that NUG1 upregulated in *P. perna* hepatopancreas likely compensates for the RCL1 depletion to maintain ribosome biogenesis fronting disturbances caused by the Vibrio injection in mussels.

It is widely known that histones are structural components of chromatin, also playing crucial roles in DNA processes (Nikapitiya et al., 2013). However, histones or histone-derived peptides have been also reported to have antimicrobial activity, since their N-terminus correspond to antimicrobial peptides (AMPs) (Nikapitiya et al., 2013; Vizioli and Salzet, 2002). Therefore, in addition to their first known function, histones participate in innate immune responses through multiple mechanisms of action both in invertebrates and vertebrates (Nikapitiya et al., 2013; Vizioli and Salzet, 2002). Free histones can be found out of the nucleus (e.g., cytoplasm, membranes, and extracellular fluids) to be easier to interact with pathogens (Nikapitiya et al., 2013). Histones bound to lipid droplets may be released into the extracellular environment or, in an alternative process, form extracellular traps (ETs) after hemocyte cell death to catch microorganisms (Nikapitiya et al., 2013). As histones also have LPS-binding activity, they may be present on the hemocyte surface functioning as PRRs (Nikapitiya et al., 2013). Additionally, histones and their fragments, combined with other molecules, can be released in the hemolymph to act as free AMPs or PRRs (Nikapitiya et al., 2013). Indeed, histone H2A has been shown as a precursor of antimicrobial peptides in several mollusks (De Zoysa et al., 2009; Li et al., 2007; Sathyan et al., 2012). Therefore, histone H2A downregulated in *P. perna* hepatopancreas in response to Vibrio suggests that these proteins have a role in some sort of antimicrobial mechanism in the mussel defense.

An ABC transmembrane type-1 domain-containing protein was upregulated in *P. perna* hepatopancreas after Vibrio injection in mussels. The ATP-binding cassette (ABC) comprises a superfamily of transmembrane transport proteins highly conserved from bacteria to humans (Ogasawara et al., 2020; Sheps et al., 2004). These proteins share the same basic structure - two ATP-binding and two transmembrane domains - and present high similarity in the sequence of their ATP-binding domain (Ogasawara et al., 2020; Sheps et al., 2004). Since they are able to interact with a huge range of substrates, ABC transporters present diverse functions (Ogasawara et al., 2020; Sheps et al., 2004). Among the four types of ABC proteins, type 1 seems to be one of the most ancient in evolution (Ogasawara et al., 2020). ABC transmembrane type-1 proteins are specialized in importing specific nutrients (Ogasawara et al., 2020) and their upregulation in *P. perna* hepatopancreas suggests some role of cellular importing fronting bacterial challenge.

Two motor dyneins heavy-chain were also upregulated in mussels injected with *V. parahaemolyticus*. As opposed to axonemal dyneins that are involved in the cilia and flagella beating, cytoplasmatic dyneins are one of the three families of cytoskeletal motor proteins (Kardon and Vale, 2009; Roberts et al., 2013). Regarding structure, the cytoplasmic dynein heavy chain consists primarily of a C-terminal motor domain and an N-terminal tail domain. The motor domain comprises six AAA domains arranged in a ring that can bind ATP and force movement along cytoskeletal filaments (Kardon and Vale, 2009; Roberts et al., 2013). The tail domain, in turn, mediates cargo interactions

directly or by recruiting accessory proteins (Kardon and Vale, 2009; Roberts et al., 2013). Therefore, dyneins are able to move along the microtubules for minus end-directed transport, that is, driving to the microtubule organizing center close to the nucleus (Kardon and Vale, 2009; Roberts et al., 2013). Dynein cargoes include organelles, vesicles, transcription factors, cytoskeletal filaments, mRNPs, among others (Kardon and Vale, 2009; Roberts et al., 2013). Hence, cytoplasmic dyneins are involved in the vast majority of cellular processes, including transmitting signals between cells or different parts of the cell for immune defense (Kardon and Vale, 2009; Roberts et al., 2013).

The armadillo repeat-containing protein 3 (ARMC3) was upregulated in Vibrio-challenged *P. perna*. Armadillo repeat-containing proteins (ARMCs) compose a family with a domain consisting of tandem repeats of approximately 42 amino acids, a structure that overall allows protein-protein binding (Gul et al., 2017; Huang et al., 2021; Tewari et al., 2010). ARMCs can also contain additional domains in the N- or C-terminus and, therefore, present additional functions related to these domains (Gul et al., 2017; Huang et al., 2021; Tewari et al., 2010). This is the case of ARMC3, which in metazoans also presents an EDR1 additional domain (Gul et al., 2017). ARMC3 is found from early metazoans to human beings, although the identity between ARMC3 in nonmammals vertebrates and *Homo sapiens* is low (49%) (Huang et al., 2021). Hence, it can present different functions among these groups. As far as known, ARMC3 takes part in cilio/flagellogenesis in animals (Lonergan et al., 2012; Pausch et al., 2016). Possibly, ARMC3 upregulation in *P. perna* suggests a rise in cilia activity demand on basophilic cells of the hepatopancreas, probably related to the secretory role of these cells. Additionally, it corroborates with the cilia-related proteins β-Tubulin and EFHC1, also upregulated in Vibrio-challenged mussels (discussed in the prior topic 3.1.2).

As discussed in the prior section (3.1.2), all the alterations associated with defense spend a lot of energy (Brokordt et al., 2019). As expected, an enzyme related to energy production and the metabolism was upregulated in Vibrio-challenged mussels compared to controls. The mitochondrial enzyme malate dehydrogenase isoform 2 (MDH2) catalyzes the reversible conversion of malate to oxaloacetate in the citric acid cycle (Nelson and Cox, 2017). Additionally, MDH2 takes part in the malate-aspartate shuttle, which acts in molecule traffic between cytosol and mitochondria to integrate different metabolic pathways (Nelson and Cox, 2017). Besides participating in the citric acid cycle, oxaloacetate is an intermediate for gluconeogenesis in the cytosol (Nelson and Cox, 2017). Further, several amino acids are deaminated in the mitochondria to be converted into oxaloacetate and then, enter the gluconeogenesis pathway (Nelson and Cox, 2017). However, to be transported by the shuttle from mitochondria to cytosol, oxaloacetate has to be reduced to malate by MDH2 and then, oxidated into oxaloacetate again (Nelson and Cox, 2017). Therefore, MDH2 upregulated suggests an increase in energy demand and, possibly, the activation of the gluconeogenesis pathway.

Mitochondrial-processing peptidase subunit beta (PMPCB) together with the subunit alpha (PMPCA) form mitochondrial processing proteases (MPPs), which act n protein targeting from the cytosol to mitochondria (Gakh et al., 2002). Hence, proteins destined for the mitochondrial matrix are targeted as precursor polypeptides carrying N-terminal prolongations (Gakh et al., 2002). Once these presequences reach their final

location within the mitochondrion, these prolongations are cleaved off by MPPs (Gakh et al., 2002). For this, PMPCA recognizes and binds to the substrate whereas PMPCB is the catalytic subunit (Gakh et al., 2002). Therefore, PMPCB upregulated in Vibrio-challenged mussels suggests an increase in protein supply for mitochondrion. Indeed, together with MDH2 upregulation, it indicates the rise in mitochondrion activity and, perhaps, an increase in protein supply to be forwarded to the gluconeogenesis pathway.

To recap the above discussion, **Figure 6** shows a frame of the immune-related roles of the altered proteins - downregulated or upregulated - in *P. perna* mussel after the *V. parahaemolyticus* challenge. As in the mussels injected with *S. enterica*, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) was upregulated. In this protein, the LRR domain typifies a toll-like receptor (TLR) for pathogens recognition and the GK domain seems to act on signal transduction. Thereafter, a rhomboid protease (RHBDL1_2_3) seems to participate in signal transduction, triggering some pathway likely related to transcriptional response against pathogens. The transcription factor hepatocyte nuclear factor-4α (NR2A1) regulates the transcription of genes related to hepatic functions and lipid metabolism. The protein THOC1 also acts on transcription and mediates mRNA export to the cytoplasm. However, before mRNA export, the proteins splicing factor 3A subunit 1 (SF3A1) and poly-U-binding factor 60 kDa (PUF60) take part in mRNA processing. In cooperation, the proteins RNA 3-phosphate cyclase-like 1 (RCL1) and nuclear GTP-binding protein (NUG1) participate in ribosome biogenesis. Thereafter, mRNA can be translated at the cytoplasm by ribosomes structurally constituted by the 60S ribosomal protein L18a (RPL18A). The mitochondrial-processing peptidase subunit beta (PMPCB) takes part in protein supply and targeting from the cytosol to mitochondria, indicating increased activity of this organelle. Corroborating with PMPCB function, the mitochondrial enzyme malate dehydrogenase isoform 2 (MDH2) (not shown in **Figure 6**) takes part in the citric acid cycle and acts in molecule traffic between cytosol and mitochondria. ABC transmembrane type-1 domain-containing protein (ABC) is specialized in importing specific nutrients, presumably critical for fronting bacteria. Histone H2A, besides its known nuclear function, may be found outside of the nucleus acting as a humoral effector via multiple mechanisms of action, because of its antimicrobial activity. Finally, the proteins β-Tubulin, EFHC1, and ARMC3 constitute cilia structure, which is probably related to the protein secretion function of the ciliated basophilic cells of the hepatopancreas.

Fig 6. Immune-related role of the altered proteins - downregulated or upregulated - in a ciliated hepatopancreatic cell of *Perna perna* mussel, 24 h post-*Vibrio parahaemolyticus* injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). As in the mussels injected with *S. enterica*, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) presents an LRR domain, which typifies a toll-like receptor (TLR) for pathogens recognition, and the GK domain, that seems to perform signal transduction. Thereafter, a rhomboid protease (RHBDL1_2_3) seems to participate in signal transduction, triggering some pathway likely related to transcriptional response against pathogens. Then, the transcription factor hepatocyte nuclear factor-4α (NR2A1) regulates the transcription of genes

related to hepatic functions and lipid metabolism. THOC1 also acts on transcription and mediates mRNA export to the cytoplasm. Nevertheless, before mRNA export, the proteins splicing factor 3A subunit 1 (SF3A1) and poly-U-binding factor 60 kDa (PUF60) take part in mRNA processing. In concert, the proteins RNA 3-phosphate cyclase-like 1 (RCL1) and nuclear GTP-binding protein (NUG1) are required for ribosome biogenesis. Then, mRNA can be translated at the cytoplasm by ribosomes constituted by the 60S ribosomal protein L18a (RPL18A). The mitochondrial-processing peptidase subunit beta (PMPCB) takes part in protein supply and targeting from the cytosol to mitochondria, indicating increased activity of this organelle. ABC transmembrane type-1 domain-containing protein (ABC) is specialized in importing specific nutrients, presumably important for fronting bacteria. Histone H2A, besides its known nuclear function, may be found out of the nucleus acting as a humoral effector via multiple mechanisms of action, because of its antimicrobial activity. Finally, the proteins β-Tubulin, EFHC1, and ARMC3 constitute cilia structure, which is probably related to the protein secretion function of the ciliated basophilic cells of the hepatopancreas.

Table 1. List of proteins significantly different from both non-injected (NC) and injected (IC) controls, identified in the hepatopancreas of *Perna perna* mussels after bacterial challenge - *Escherichia coli* (EC), *Salmonella enterica* (SE), and *Vibrio parahaemolyticus* (VP).

Protein intensity (Log_2)					Protein ID (*Mytilus coruscus*)	Protein name	COG	GO Term of Biological process	Functional role discussed
NC	**IC**	**EC**	**SE**	**VP**					
2.38726 (a)	1.3484 (a)	2.10945 (a)	0 (a,b)	-2.38726 (b)	A0A6J8EX32	RCL1	A	GO:0042254	IR: Ribosomal structure and translation
-2.62858 (a)	-1.47506 (a)	-1.37232 (a)	0 (a,b)	2.62858 (b)	A0A6J7ZVU2	THOC1	Y	GO:0007165	IR: Transcription and processing
-1.60211 (a)	-2.93367 (a)	-2.59738 (a)	0 (a,b)	2.93367 (b)	A0A6J8AQ94	MDH2 (EC 1.1.1.37)	C	GO:0019752	EPM
-1.74661 (a)	-2.12859 (a)	-2.11956 (a)	0 (a,b)	2.12859 (b)	A0A6J8AVB3	HNF4α or NR2A1	K	GO:0055088	IR: Transcription and processing
-1.96165 (a)	-2.9873 (a)	-2.95419 (a)	0 (a,b)	2.9873 (b)	A0A6J8AVI6	ARMC3	U	-	IR: Cytoskeleton-associated response
-1.83666 (a)	-1.99424 (a)	-1.85217 (a)	0 (a,b)	1.99424 (b)	A0A6J8BYT8	PMPCB (EC 3.4.24.64)	O	GO:0006627	IR: Protein processing
-1.73032 (a)	-2.61043 (a)	-1.48601 (a)	0 (a,b)	2.61043 (b)	A0A6J8DLP9	NUG1	O	GO:0042254	IR: Ribosomal structure and translation
-2.20849 (a)	-2.90543 (a)	-3.19666 (a)	0 (a,b)	3.19666 (b)	A0A6J8EP08	DNAH	Z	-	IR: Cytoskeleton-associated response
-1.79806 (a)	-2.75469 (a)	-2.22081 (a)	0 (a,b)	2.75469 (b)	A0A6J8ETV0	DNAH	Z	-	IR: Cytoskeleton-associated response
-2.57474 (a)	-2.46286 (a)	-2.64498 (a)	0 (a,b)	2.64498 (b)	A0A6J8EX85	Uncharacterized protein	-	-	-
-1.53336 (a)	-2.23568 (a)	0 (a,b)	2.23568 (b)	1.95547 (b)	A0A6J8B1G4	β-Tubulin	Z	GO:0005200	IR: Cytoskeleton-associated response

-1.84442 (a)	-1.87709 (a)	0 (a,b)	1.87709 (b)	1.33182 (b)	A0A6J8C231	LRGUK	FT	GO:0016310	IR: Recognition and signal transduction
-2.08 (a)	-2.38518 (a)	0 (a,b)	2.08434 (b)	2.38518 (b)	A0A6J8DBD6	EFHC1	S	GO:0000226	IR: Cytoskeleton-associated response
-2.11222 (a)	-1.8169 (a)	0 (a,b)	2.11222 (b)	2.01685 (b)	A0A6J8DKT3	RPL18A	J	GO:0006412	IR: Ribosomal structure and translation
1.96583 (a)	1.81292 (a)	0 (a,b)	-1.94087 (b)	-1.96583 (b)	A0A6J8DBY2	SF3A1	A	GO:0045292	IR: Transcription and processing
-1.85751 (a)	-2.69884 (a)	0 (a,b)	2.69884 (b)	0 (a,b)	A0A6J8B4X1	OGDHL (EC 1.2.4.2)	C	GO:0004591	EPM
-1.67409 (a)	-2.1063 (a)	0 (a,b)	2.1063 (b)	0 (a,b)	A0A6J8BFT8	GPD2 (EC 1.1.5.3)	C	GO:0052590	EPM
-1.99897 (a)	-2.09925 (a)	0 (a,b)	2.09925 (b)	0 (a,b)	A0A6J8BY71	MTFP1	S	GO:0000266	IR: Apoptotic process
-1.63546 (a)	-1.74695 (a)	0 (a,b)	1.74695 (b)	0 (a,b)	A0A6J8C994	DNAH9	Z	GO:0003341	IR: Cytoskeleton-associated response
-2.17627 (a)	-2.00809 (a)	0 (a,b)	2.17627 (b)	0 (a,b)	A0A6J8D105	DNAH3	Z	GO:0000003	IR: Cytoskeleton-associated response
2.3344 (a)	2.41264 (a)	0 (a,b)	-2.41264 (b)	2.11245 (a)	A0A6J8DAJ1	GALK2 (EC 2.7.1.157)	G	GO:0004335	EPM
-2.00612 (a)	-1.56804 (a)	0 (a,b)	2.00612 (b)	-1.43933 (a)	A0A6J8BGT0	PA28γ or PSME3	O	GO:0008537	IR: Protein processing
-2.16034 (a)	-3.12093 (a)	0 (a,b)	3.12093 (b)	-1.45282 (a)	A0A6J8CT60	Cilia- and flagella-associated protein 299	S	GO:0003341	IR: Cytoskeleton-associated response
1.83903 (a)	2.5799 (a)	0 (a,b)	1.6229 (a)	-2.5799 (b)	A0A6J8EST1	Histone H2A	B	GO:0006325	IR: Humoral effector
-2.0011 (a)	-1.55342 (a)	0 (a,b)	-2.29772 (a)	2.29772 (b)	A0A6J8AYV2	PUF60	A	GO:0003723	IR: Transcription and processing
-1.46088 (a)	-2.82524 (a)	0 (a,b)	-1.92149 (a)	2.82524 (b)	A0A6J8BVY9	ABC transmembrane type-1 domain-containing	Q	GO:0055085	IR: Transmembrane

						protein			transport
-1.77553 (a)	-2.25292 (a)	-2.05586 (a)	2.25292 (b)	0 (a,b)	A0A6J8DLC4	Stress-activated protein kinase JNK (EC 2.7.11.24)	T	GO:0006468	IR: Recognition and signal transduction
2.70474 (a)	3.16862 (a)	-1.89236 (b,c)	-3.16862 (b)	2.37932 (a,c)	A0A6J8C596	FERM domain-containing protein	T	-	IR: Signal transduction and cytoskeleton-associated response
2.05026 (a)	2.00156 (a)	-2.24928 (b)	0 (a,b)	2.24928 (a)	A0A6J8E5Z8	vWA domain-containing protein	S	-	IR: Humoral effector
1.5336 (a,c)	1.60073 (a,c)	-1.39648 (b,c)	-3.14933 (b)	3.14933 (a)	A0A6J8BRN5	ACTB_G1	Z	GO:0048870	IR: Cytoskeleton-associated response
2.04012 (a)	2.34246 (a)	0 (a,b)	0 (a,b)	-2.34246 (b)	A0A6J8EM30	RHBDL1_2_3 (EC 3.4.21.105)	T	GO:0004252	IR: Recognition and signal transduction

EPM - Energy production and metabolism; IR – Immune response

3.2 Specific response to *Vibrio parahaemolyticus*

Mussels challenged with *Vibrio parahaemolyticus* (VP) presented 343 proteins significantly different from all the other conditions (NC - non-injected control; IC - injected control; EC - *Escherichia coli*; and SE - *Salmonella enterica*), within a total of 597 proteins that significantly differed from at least one condition. Among these 343 proteins, 327 were downregulated whereas only 16 were upregulated (**Figure 7.a**). These altered proteins in specific response to Vibrio were classified into functional categories based on the Database of Clusters of Orthologous Genes (COGs) and the most frequent (%) COGs among them are shown in **Figure 7.b**, as follows: T - Signal transduction mechanisms (13%); O - Posttranslational modification, protein turnover, chaperones (12%); S - Function unknown (10%); U - Intracellular trafficking, secretion, and vesicular transport (9%); A - RNA processing and modification (6%); Z - Cytoskeleton (6%); K - Transcription (6%); J - Translation, ribosomal structure, and biogenesis (5%); and remaining categories (33%). The 16 upregulated proteins did not fit in any COG functional category, therefore significantly different proteins in specific response to Vibrio of all the above COG categories were downregulated.

Fig 7. a) Number of proteins downregulated (n= 327) and upregulated (n= 16) in *Perna perna* mussel among the 343 proteins significantly different between *Vibrio parahaemolyticus* (VP) and all the others conditions (NC - non-injected control; IC - injected control; EC - *Escherichia coli*; and SE - *Salmonella enterica*). **b)** Frequency (%) of functional categories of the proteins altered after VP challenge, which are significantly different from all other conditions, based on the Database of Clusters of Orthologous Genes - COGs. T - Signal transduction mechanisms; O - Posttranslational modification, protein turnover, chaperones; S - Function unknown; U - Intracellular trafficking, secretion, and vesicular transport; A - RNA processing and modification; Z - Cytoskeleton; K - Transcription; J - Translation, ribosomal structure, and biogenesis. As the 16 upregulated proteins did not fit in any COG functional category, significantly different proteins in specific response to VP in all functional categories shown in the graph were downregulated.

Figure 8 compares the percentage (%) of proteins by each one of the eight most frequent COGs, between proteins significantly different (downregulated) only for mussels challenged by *V. parahaemolyticus* (VP) and the total of proteins found in *P. perna* mussels (all the other condition-groups). In the specific response to Vibrio, that is, proteins significantly different from all the other conditions, there was a lower percentage of proteins fitted in the categories cytoskeleton (Z) and function unknown (S) compared with the total of proteins found (**Figure 8**). On the other hand, the percentage of proteins included in the categories transcription (K), RNA processing and modification (A), and posttranslational modification, protein turnover, and chaperones (O) was smoothly higher than in the total of proteins found in *P. perna* (**Figure 8**). Although the functional categories translation, ribosomal structure and biogenesis (J), signal transduction mechanisms (T), and intracellular trafficking, secretion, and vesicular transport (U) comprise one of the main portions of significantly different proteins in specific response to Vibrio, the percentage of proteins fitted into these categories did not change quantitatively compared with the total of proteins found (**Figure 8**).

Fig 8. Comparison of the percentage (%) of proteins between all groups (total proteins found in *Perna perna* mussels) and proteins altered in specific response to *Vibrio parahaemolyticus* (VP; significantly different from all the other groups), according to the eight most frequent COG functional categories.

Unlike most marine bacteria, the genus *Vibrio* comprises multiple species harmful to shellfish (Gosling, 2015). Indeed, vibrioses constitute most reports of bacterial diseases of bivalves, often leading to mass mortality (Gosling, 2015). Among the approximately 100 species in the genus, the following *Vibrio* species are often associated with diseases in fish and shellfish: *V. parahaemolyticus*, *V. vulnificus*, *V. furnissii*, *V. campbellii*, *V. harveyi*, *V. alginolyticus*, and *V. anguillarum* (Liu et al., 2019). These pathogenic strains produce several virulence factors (e.g., enterotoxins, cytotoxins, proteases, among others), which are crucial to their invasive mechanisms and subsequent infection (Liu et al., 2019). In fact, the proteomic response of *P. perna* specifically to *V. parahaemolyticus* was more substantial (343 altered proteins) than that to the marine introduced bacteria - *E. coli* and *S. enterica*.

Since bivalves throve in a rich-Vibrio environment throughout their evolutionary history, they likely assembled countless pattern recognition receptors (PRRs) to identify conserved pathogen-associated molecular patterns (PAMPs) of *Vibrio* species (Gosling, 2015; Liu et al., 2019). The first phase for generating a defense response is recognition and signal transduction (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). However, it can be impaired as the pathogen virulence increases (Liu et al., 2019; Yu et al., 2019). Indeed, the majority of significantly different proteins of *P. perna* in specific response to Vibrio fitted in the COG T - signal transduction mechanisms. Nevertheless, Vibrio seems to suppress *P. perna* immune response since among the 343 altered proteins in specific response to *V. parahaemolyticus*, 326 proteins were downregulated (**Figure 7**). These proteins were directly or indirectly related to defense, including proteins comprised in the COG T. Accordingly, this result suggests that Vibrio is virulent for *P. perna* mussels and likely suppresses key-proteins related to immune response.

Vibrio parahaemolyticus was also found to be highly virulent for surf clams *Paphia undulate* (Yu et al., 2019). The rate of survival of the clams abruptly declined at 24 h (87.2%) and 48 h (65.3%) post-infection with *V. parahaemolyticus* (Yu et al., 2019). Additionally, the clams presented an extensive range of downregulated genes (1433), which were associated with cellular and molecular mechanisms for pathogen recognition, and immunity triggering (Yu et al., 2019). The razor clam *Sinonovacula constricta* also presented a strong response to *V. parahaemolyticus* infection (Zhao et al., 2017). After injection, 1,781 significant differentially expressed transcripts were obtained in the gills and 490 in the hepatopancreas (Zhao et al., 2017). Several transcripts in both tissues were specifically related to immune response, such as pathogen recognition receptors (PRRS) and immune signaling and cell communication (Zhao et al., 2017). Corroborating these results, proteomic studies with other *Vibrio* species (e.g., *V. anguillarum* and *V. tapetis*) injected in the mussel *Mytilus galloprovincialis* and the Manila clam *Ruditapes philippinarum* have also altered several proteins related to recognition and signaling, among others immune roles (Ji et al., 2013; Smits et al., 2020; Wu et al., 2013).

After recognition and signaling, the response at the subcellular level involves the transcription of response genes and RNA processing, including splicing to obtain alternative mRNAs (Gerdol and Venier, 2015; Hatanaka et al., 2009; Irazoqui et al., 2010; Sousa and Hinzmann, 2020). Then, the translation and posttranslational modifications of recent-constructed proteins occur to make them functional and targeted (Gerdol and Venier, 2015; Hatanaka et al., 2009; Irazoqui et al., 2010; Sousa and Hinzmann, 2020). These recent-formed molecules, in turn, perform different roles at several levels to trigger both cellular and humoral immune responses, including a huge range of recognition factors and humoral effectors (Gerdol and Venier, 2015; Sousa and Hinzmann, 2020). The main functional categories of proteins significantly different (downregulated) in specific response to Vibrio corresponded with the above processes and reinforce Vibrio virulence to *P. perna*, namely: O - Posttranslational modification, protein turnover, chaperones (12%); A - RNA processing and modification (6%); K - Transcription (6%); J - Translation, ribosomal structure and biogenesis (5%) (**Figure 7.b**).

Last but not least, the functional categories cytoskeleton and intracellular traffic, secretion, and vesicular transport had an increased percentage of proteins downregulated in specific response of *P. perna* to Vibrio (**Figure 7.b**). The cytoskeleton is the basis of all cellular movements and works together with transporter proteins, to carry organelles, molecules, and vesicles (Kardon and Vale, 2009; Roberts et al., 2013). Consequently, the cytoskeleton is closely linked to secretion (Kardon and Vale, 2009; Roberts et al., 2013). As discussed in the prior topics (3.1.1, 3.1.2, and 3.1.3), the main role of ciliated basophilic cells of hepatopancreas is protein secretion (Tunali and Erkan, 2008; Yang et al., 2021). In front of a bacterial challenge, basophilic cells likely secret proteins and other molecules related to mussel defense, such as humoral effectors observed in other bivalves infected with *Vibrio* (Ji et al., 2013; Smits et al., 2020; Wu et al., 2013; Yang et al., 2016). Therefore, the high percentage of downregulated proteins in the hepatopancreatic cells of *P. perna*, which are involved in transcription, RNA processing, translation and protein production, cytoskeleton, and secretion, again support the hypothesis that *V. parahaemolyticus* is virulent to the mussel and impairs several crucial processes to triggering defense response.

4. Conclusion

This is the first study using a shotgun proteomic approach in the mussel *Perna perna* after bacterial challenge. Therefore, for the first time, it is possible to notice a global profile of *P. perna* hepatopancreas proteins (a total of 3,805), focusing on critical proteins for the mussel-bacteria relationship. As expected, mussels injected with the marine indigenous bacterium *V. parahaemolyticus* presented a more prominent response - 343 proteins significantly different from all the other conditions, from a total of 597 significantly different - than those challenged with marine introduced bacteria - *E. coli* and *S. enterica*. Additionally, as the large majority of proteins in specific response to *V. parahaemolyticus* were downregulated (327), this bacterium seems to be virulent to the mussel *P. perna* since it suppresses mussel's immune response. In general, that is, for the three challenge bacteria, significantly different proteins - downregulated or upregulated - were found to perform critical roles in immune response at all levels in the mussel hepatopancreas, as follows: recognition and signal transduction; transcription;

RNA processing; translation and protein processing; secretion; and humoral effectors against bacteria. Hence, this study was the first step to better understanding *P. perna* immune responses at molecular levels and pointing out critical and strategic proteins against bacteria. Going beyond, these proteins can be helpful in biotechnology as molecular biomarkers related to bacteria exposure. Then, it can be applied in environmental monitoring and to assess the health status and/or food safety of commercial species (such as the brown mussel *P. perna*). For example, the early detection of *Vibrio* species in mussel farming and its treatment. Additionally, among the huge range of antimicrobial compounds in bivalves, it can be found bioactive molecules of human interest. However, further studies are needed to go deeper into this fertile field and explore promising molecules to be applied in several areas, including coastal marine resource management such as bivalve farms.

Acknowledgements

The authors are grateful to Dr. Rosana Barreto Rocha Ferreira (UFRJ), Dr. Júlia Peixoto de Albuquerque (UFF) and Dr. Renata Garcia Costa (Fiocruz), for providing the bacteria strains used in this work. The authors thank Mr. Ivanildo Chagas dos Santos and Débora Silva dos Santos, the first author's father and sister respectively, and Professor Luciano N. Santos for assisting with mussels sampling. The authors also thank Dr. Leandro Machado Rocha, MSc. Jorge Andrés Duarte Duarte and the technician Camila Soares Silvestre Toledo from the faculty of pharmacy (UFF) for helping with sample lyophilization. This work was supported by National Council for Scientific and Technological Development – CNPq [Fernanda Silva dos Santos – Sandwich doctoral fellowship 203434/2020-0] and by ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015) and co-funded by a grant from the French government under the program "Investissements d'Avenir" embedded in France 2030. Additionally, this study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 – [Fernanda Silva dos Santos – doctoral fellowship 88882.457000/2019.1], and financially supported by the Universidade Federal do Estado do Rio de Janeiro (UNIRIO) – [INOVA UNIRIO IN-01/2019 attributed to Natascha Krepsky].This study is also part of the project "PELD/ Guanabara: Pesquisas Ecológicas de Longa Duração" financially supported by Brazilian National Council for Scientific and Technological Development (CNPq; 558083/2009-9 and 441373/2016-0) and Foundation Carlos Chagas Filho Research Support of the State of Rio de Janeiro (FAPERJ; 136375 and E-26/110.144/2013). Raquel A. F. Neves is grateful to the research grant attributed by FAPERJ (E-26/201.283.2021).

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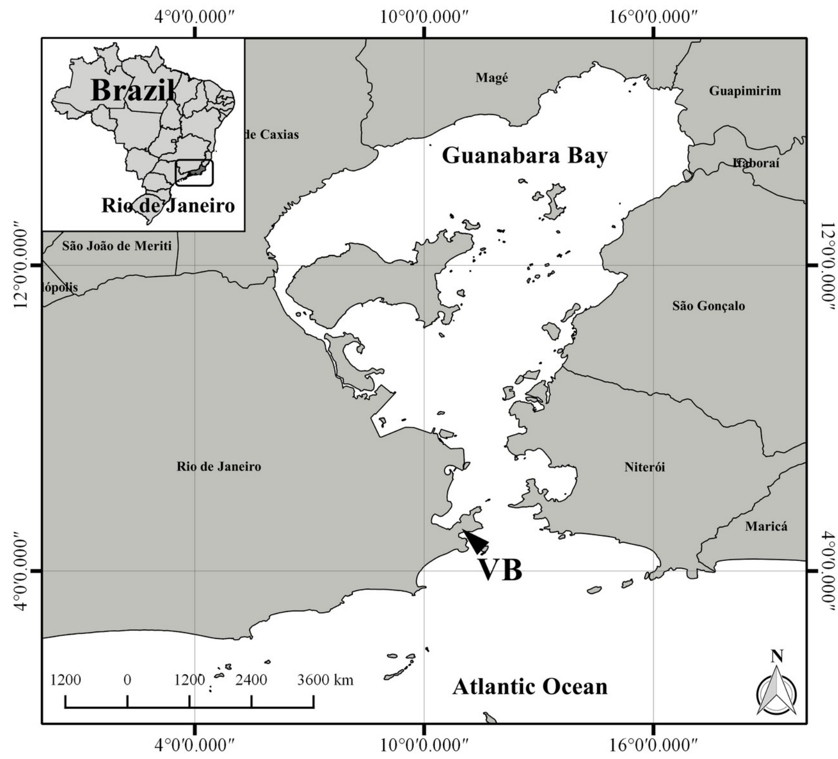

Fig 1. Vermelha Beach (VB), Rio de Janeiro, Brazil: mussels and seawater sampling location.

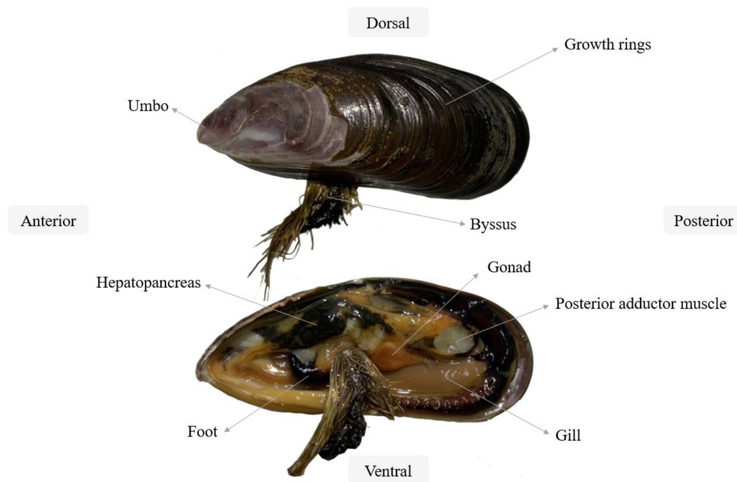

Fig 2. The external and internal anatomical features of the mussel *Perna perna*.

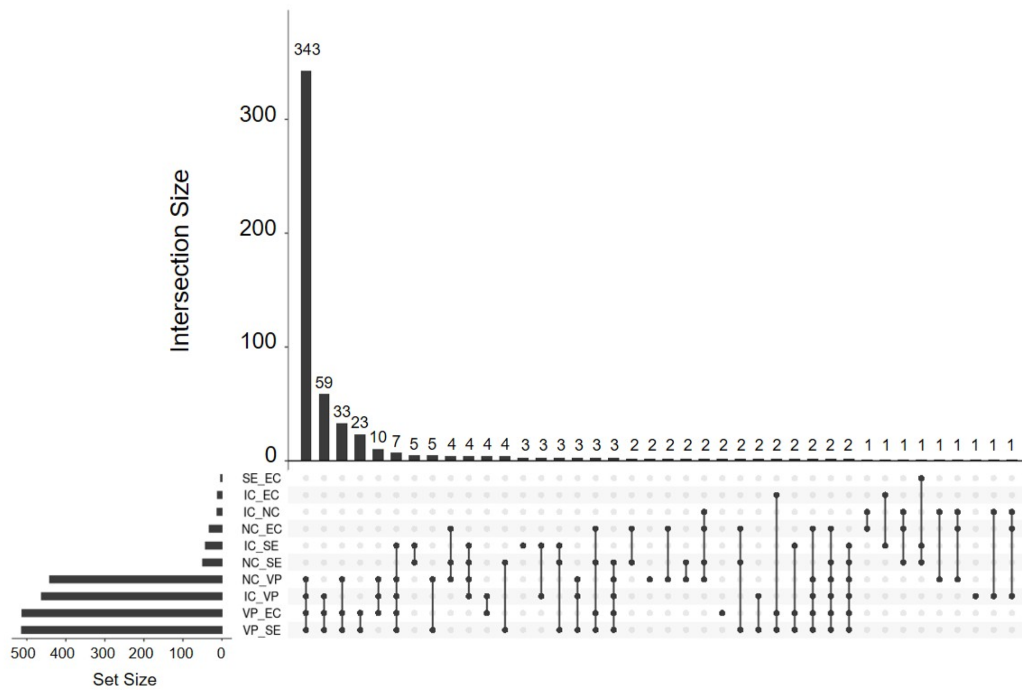

Fig 3. Upset plot for visualizing intersecting sets of significantly different proteins among the tested conditions. NC - non-injected control; IC - injected control; EC - *Escherichia coli*; SE - *Salmonella enterica*; and VP - *Vibrio parahaemolyticus*. SE_EC means a significant difference between these conditions and so on.

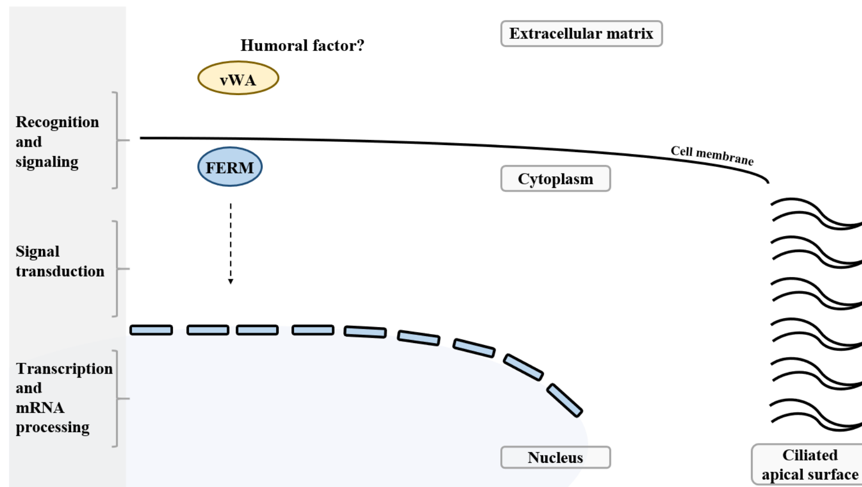

Fig 4. Immune-related role of the altered proteins in a ciliated hepatopancreatic cell of Perna perna mussel, 24h post-Escherichia coli injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). In mussels challenged with E. coli, a von Willebrand Factor A domain-containing protein (vWA) and a FERM domain-containing protein (FERM) were downregulated. FERM is probably located close to the plasma membrane and takes part in signal transduction. VWA seems to act as a humoral effector in mollusks, primarily in the extracellular matrix.

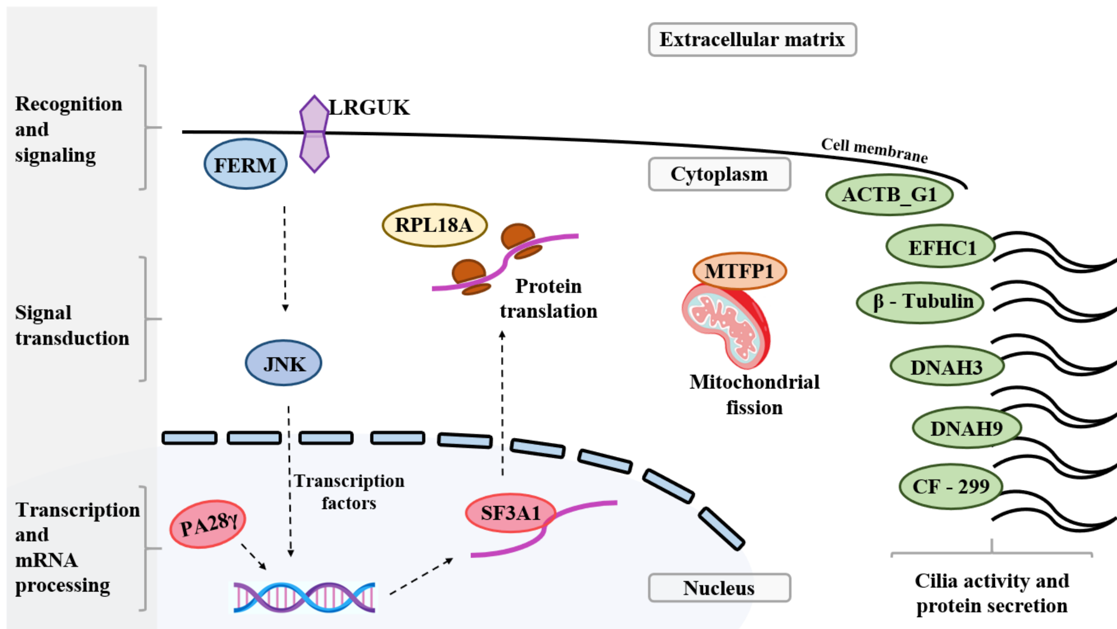

Fig 5. Immune-related role of the altered proteins - downregulated or upregulated - in a ciliated hepatopancreatic cell of Perna perna mussel, 24 h post-Salmonella enterica injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). First of all, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) acts on pathogens recognition and signal transduction, through the LRR and GK domains, respectively. As in the mussels injected with E. coli, the FERM domain-containing protein (FERM) probably also takes part in signal transduction close to the plasma membrane. Afterward, the stress-activated protein kinase JNK (JNK) participates at the end of the mitogen-activated protein kinase (MAPK) cascade, phosphorylating transcription factors that regulate the transcription of response genes. The proteasome activator PA28γ (PA28γ) located in the nucleus is also presumably involved in transcription or apoptosis. After transcription, the splicing factor 3A subunit 1 (SF3A1) takes part in mRNA processing, which is translated at the cytoplasm by ribosomes constituted by the 60S ribosomal protein L18a (RPL18A). Mitochondrial fission process protein 1 (MTFP1) mediates mitochondrial fission, which rises under cellular stress and during apoptosis. Last but not least, the proteins actin beta/gamma 1 (ACTB_G1), Tubulin beta chain (β-Tubulin), axonemal dyneins (DNAH3 and DNAH9), EFHC1, Cilia- and flagella-associated protein 299 (CF-299) constitute cytoskeleton and cilia structure, which are probably related to the protein secretion function of the ciliated basophilic cells of hepatopancreas.

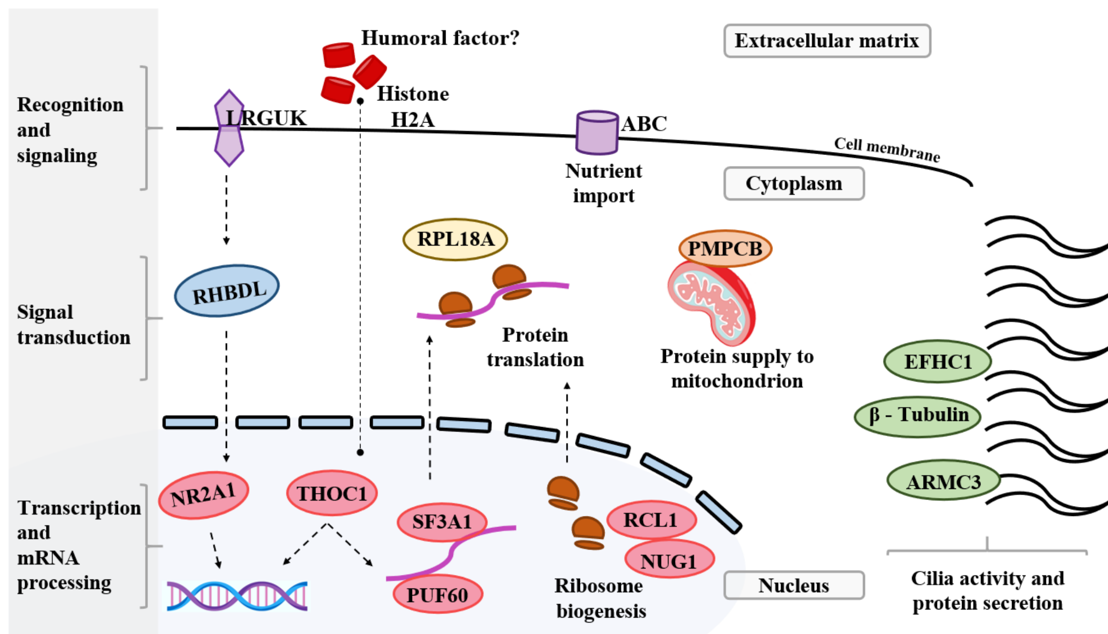

Fig 6. Immune-related role of the altered proteins - downregulated or upregulated - in a ciliated hepatopancreatic cell of Perna perna mussel, 24 h post-Vibrio parahaemolyticus injection. Herein, the primary steps for initiating an immune reaction against pathogens, according to the cell compartment, are displayed as: recognition and signaling (cell periphery); signal transduction (cytoplasm); and transcription and mRNA processing (nucleus). As in the mussels injected with S. enterica, the leucine-rich repeat (LRR) and guanylate kinase (GK) domain-containing protein (LRGUK) presents an LRR domain, which typifies a toll-like receptor (TLR) for pathogens recognition, and the GK domain, that seems to perform signal transduction. Thereafter, a rhomboid protease (RHBDL1_2_3) seems to participate in signal transduction, triggering some pathway likely related to transcriptional response against pathogens. Then, the transcription factor hepatocyte nuclear factor-4α (NR2A1) regulates the transcription of genes related to hepatic functions and lipid metabolism. THOC1 also acts on transcription and mediates mRNA export to the cytoplasm. Nevertheless, before mRNA export, the proteins splicing factor 3A subunit 1 (SF3A1) and poly-U-binding factor 60 kDa (PUF60) take part in mRNA processing. In concert, the proteins RNA 3-phosphate cyclase-like 1 (RCL1) and nuclear GTP-binding protein (NUG1) are required for ribosome biogenesis. Then, mRNA can be translated at the cytoplasm by ribosomes constituted by the 60S ribosomal protein L18a (RPL18A). The mitochondrial-processing peptidase subunit beta (PMPCB) takes part in protein supply and targeting from the cytosol to mitochondria, indicating increased activity of this organelle. ABC transmembrane type-1 domain-containing protein (ABC) is specialized in importing specific nutrients, presumably important for fronting bacteria. Histone H2A, besides its known nuclear function, may be found out of the nucleus acting as a humoral effector via multiple mechanisms of action, because of its antimicrobial activity. Finally, the proteins β-Tubulin, EFHC1, and ARMC3 constitute cilia structure, which is probably related to the protein secretion function of the ciliated basophilic cells of the hepatopancreas.

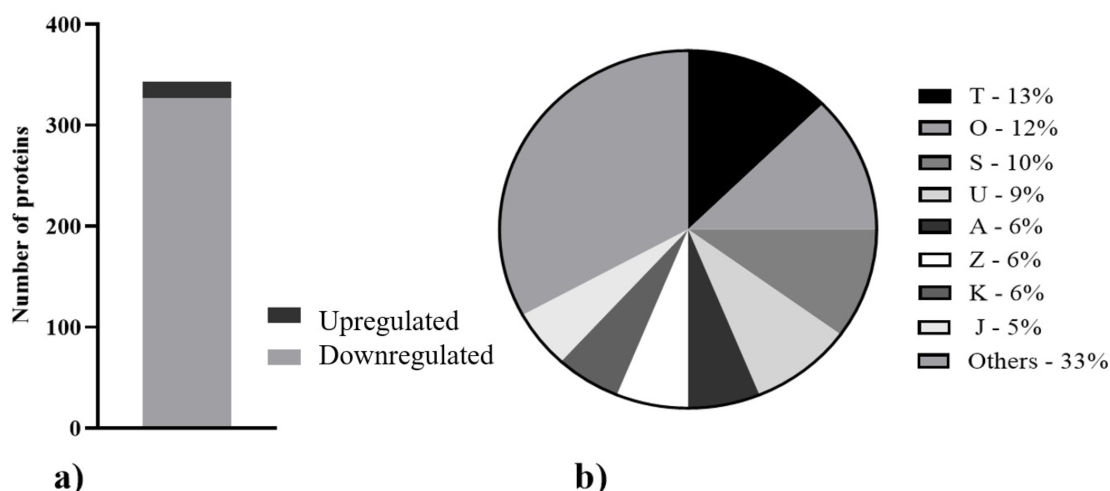

Fig 7. a) Number of proteins downregulated (n= 327) and upregulated (n= 16) in Perna perna mussel among the 343 proteins significantly different between Vibrio parahaemolyticus (VP) and all the others conditions (NC - non-injected control; IC - injected control; EC - Escherichia coli; and SE - Salmonella enterica). **b)** Frequency (%) of functional categories of the proteins altered after VP challenge, which are significantly different from all other conditions, based on the Database of Clusters of Orthologous Genes - COGs. T - Signal transduction mechanisms; O - Posttranslational modification, protein turnover, chaperones; S - Function unknown; U - Intracellular trafficking, secretion, and vesicular transport; A - RNA processing and modification; Z - Cytoskeleton; K - Transcription; J - Translation, ribosomal structure, and biogenesis. As the 16 upregulated proteins did not fit in any COG functional category, significantly different proteins in specific response to VP in all functional categories shown in the graph were downregulated.

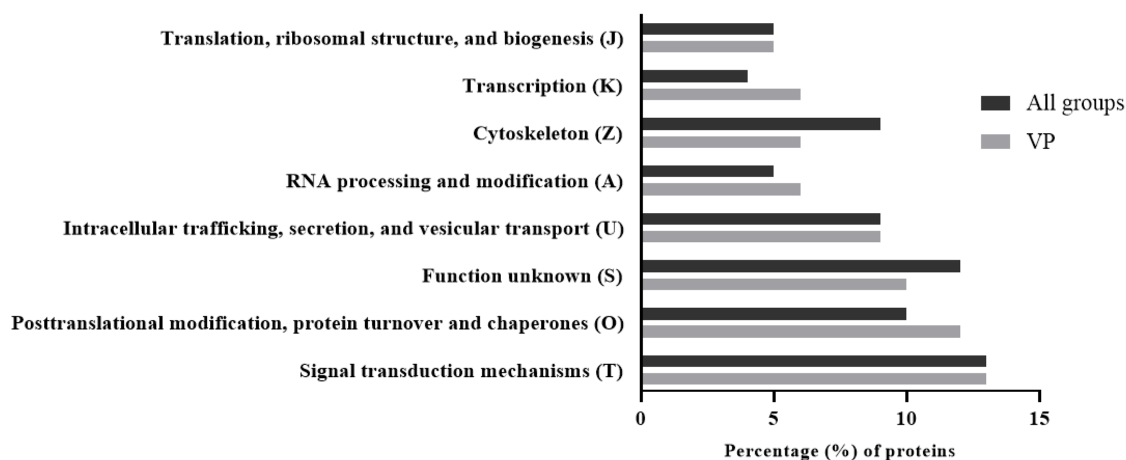

Fig 8. Comparison of the percentage (%) of proteins between all groups (total proteins found in Perna perna mussels) and proteins altered in specific response to Vibrio parahaemolyticus (VP; significantly different from all the other groups), according to the eight most frequent COG functional categories.