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Finding immune gene expression differences induced by marine bacterial pathogens in the Deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*

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Abstract. The deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* lives in a natural environment characterised by extreme conditions of hydrostatic pressure, temperature, pH, high concentrations of heavy metals, methane and hydrogen sulphide. The deep-sea vent biological systems represent thus the opportunity to study and provide new insights into the basic physiological principles that govern the defense mechanisms in vent animals and to understand how they cope with microbial infections. Hence, the importance of understanding this animal's innate defense mechanisms, by examining its differential immune gene expressions toward different pathogenic agents. In the present study, *B. azoricus* mussels were infected with single suspensions of marine bacterial pathogens, consisting of *Vibrio splendidus, Vibrio alginolyticus,* or *Vibrio anguillarum,* and a pool of these *Vibrio* bacteria. *Flavobacterium* suspensions were also used as a non-pathogenic *bacterium.* Gene expression analyses were carried out using gill samples from infected animals by means of quantitative-Polymerase Chain Reaction aimed at targeting several immune genes. We also performed SDS-PAGE protein analyses from the same gill tissues.

We concluded that there are different levels of immune gene expression between the 12 h to 24 h exposure times to various bacterial suspensions. Our results from qPCR demonstrated a general pattern of gene expression, decreasing from 12 h over 24 h post-infection. Among the bacteria tested, *Flavobacterium* is the bacterium inducing the highest gene expression level in 12 h post-infections animals. The 24 h infected animals revealed, however, greater gene expression levels, using *V. splendidus* as the infectious agent. The SDS-PAGE analysis also pointed at protein profile differences between 12 h and 24 h, particularly evident for proteins of 18–20 KDa molecular mass, where most dissimilarity was found. Multivariate analyses demonstrated that immune genes, as well as experimental infections, clustered in discrete groups in accordance with the gene expression patterns induced by bacterial pathogens.

1 Introduction

Deep-sea hydrothermal vents were discovered in the seafloor where the oceanic crust is subjected to active volcanic occurrences such as the Mid-Atlantic Ridge (Childress et al., 1992). Chemosynthetic-based ecosystems are built around the deep-sea hydrothermal vents and support large microbial communities (Teske, 2009) and symbioses between dominant fauna and intracellular bacteria permitting the existence of animals and microbes under such extreme environments (Duperron et al., 2009).

Deep-sea vent mussels of the *Bathymodiolus* genus are dominant members at hydrothermal vents and cold seep habitats. These mussels have the peculiarity of sheltering both endosymbiotic sulphide-oxidizing and methane-oxidizing bacteria in their gills (Salerno et al., 2005; De Chaine et al., 2006), thus supporting their endurance within this type of environment (Bettencourt et al., 2008). *Bathymodiolus azoricus* is the dominant species in deep-sea hydrothermal vents in the Azores region and is adapted to extreme conditions that are characterised by toxic concentrations of heavy metals, acidic pH and absence of light (Bettencourt et al., 2007; Colaço et al., 2010).

The innate immune system is the foremost line of host defense against microbial pathogens (Janeway and Medzhitov, 2002; Kumar et al., 2009). This system recognises conserved molecules of microbial origin found in bacteria, viruses, protozoa and fungi, and known as Microbial-Associated Molecular Patterns (MAMPs) (Akira and Hemmi, 2003; Medzhitov, 2007; Boudsocq et al., 2010), which elicits an initial response through host-activated Pattern Recognition Receptors (PRR) (Medzitov, 2001; Kumar et al., 2009). Consequently, the innate immune system acts to protect the individual from invasive agents by detecting molecular signatures of infection that in turn initiates effector responses (Bettencourt et al., 2010). Invertebrates and molluscan immune responses are notorious for their ability to defend themselves against bacteria, fungi, and parasites. Their first lines of defense against infectious agents are physical and chemical barriers, such as the shell and exoskeleton, and deterrent chemical compounds. Once these barriers are breached, humoral and cellular reactions are set to function through hemolymph constituents and hemocytes respectively (Galloway and Depledge, 2001). Likewise, in bivalves, cellular and humoral components are required for defense responses allowing them to overcome pathogens that are naturally present in marine environments (Labreuche et al., 2006). The main cellular immune response against pathogens in molluscan is phagocytosis (Cheng, 1981; Feng, 1988; May et al., 2001).

Vibrio bacteria are a major cause of disease occurring in the marine environment (Demírcan and Candan, 2006) causing high mortality in some bivalves (Beaz-Hidalgo et al., 2010). Although the pathologies caused by *Vibrio* in bivalves have been described since the 1960s (Paillard et al., 2004) to this day, some of these species, such as *V. alginolyticus*, *V. splendidus*, *V. anguillarum*, are still being reported in case studies. *V. alginolyticus* and *V. splendidus* cause histological lesions that affect mainly the mantle, the velum, and the connective tissue of infected organisms (Gómez-León et al., 2005). Even though *Vibrio anguillarum* is the most studied aetiological agent of vibriosis, other members of the genus *Vibrio* have been implicated in epizootics of cultured and wild marine fish and shellfish (Toranzo and Barja, 1990). The presence of *Vibrio diabolicus* has also been found at deep-sea hydrothermal vents and more recently the presence of a phenotypically related pathogenic *Vibrio* species was also reported at deep-sea vent sites (Raguénès et al., 1997; Hasan et al., 2010). Moreover, a wide phylogenetic spectrum of marine *Flavobacteria* was found in deep-sea sediments mainly oxidizing thiosulfate bacteria and several species that cause diseases in freshwater fish (Bernardet et al., 1996; Teske et al., 2000; Alain et al., 2004).

In view of this, the problem of microbial threat and the need for immunity exist in deep sea mussels, however, differences in immune gene expression in animals living in such distinct habitats, are likely to occur as well as the expression of their immune discriminatory capabilities. In this context, experimental infections carried out with *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio anguillarum* strains and *Flavobacterium*, were performed and subsequently the expression of vent mussel immune genes analysed by qPCR. Additionally, protein profiles were also analysed by SDS-PAGE. Our results suggest that there are significant differences in gene expression profiles between the immune genes studied, among several bacteria used, and over the course of time during infection.

2 Material and methods

2.1 Biological samples collection

The mussels were collected from the hydrothermal vent field Menez Gwen (850 m depth, 37°50, 8–37°51.6 N, 31°30–31°31.8 W), with the French R/V *"Pourquoi Pas?"* using the Remote Operated Vehicle (ROV Victor 6000) (MoMARSAT cruise, 28 June–22 July 2011).

2.2 Bacterial preparations and infections

B. azoricus mussels collected at the Menez Gwen field, were maintained in 20 L seawater containing plastic vessels, artificially supplemented with methane (CH_4) and dissolved Sodium Sulfide (Na_2S) (Bettencourt et al., 2010; Colaço et al., 2010). Subsequently, six groups of 8 animals each, corresponding to six distinct experimental conditions, were set into 2 L seawater containing beakers. Mussels were then, infected with a suspension of marine bacterial pathogens, consisting of one of the following strains *V. splendidus*, *V. alginolyticus*, or *V. anguillarum*, and with a pool of the 3 Vibrio strains. As yet another distinct bacterium, a suspension of *Flavobacterium* was also used. The control condition was regarded as incubations with only in seawater. Vibrios were obtained from Dr. Bassem Allam (Stony Brook University, NY, USA) and from Dr. Antonio Figueras (CSIC, IIM, Vigo, Spain). *Flavobacterium* was isolated from a marine bacterial screening from Azorean seawater samples. The marine *Flavobacterium* used in this study was identified to its genus by 16S PCR amplicon sequencing (Bettencourt unpublished data). 25 mL of bacterial inoculums were prepared from overnight cultures grown at room temperature in Marine Broth (Difco™ Marine Broth 2216) ($OD_{600} = 1.5$) and

Table 1. Forward and reverse primer sequences of the target Immune response genes according to the immune functional categories, Recognition, Signaling, Transcription and Effector genes (Bettencourt et al., 2010), used in qPCR analyses.

	Contig Reference DeepSea Database	Forward primer 5'-3'	Reverse primer 5'-3'
Housekeeping gene			
28S		AAGCGAGAAAAGAAACTAAC	TTTACCTCTAAGCGGTTTCAC
Recognition genes			
Rhamnose binding lectin	mussel_c2955	ACAATGGGTTGATTTGTTTGCCGA	CCGGGGGCCTGAAAGTTGGT
PGRP	mussel_c1910	TCACACGGAAGGAGGAGCGT	AGGGCTGCCTTGGATGGTGT
Serpin	mussel_c6158	AGGGTTGTGCGTGAAGTGGA	TCTCAAAGCGAGGCTGCCAGA
Aggrecan	mussel_lrc83347	ATAGCCATC GCCAGTCACCA	ACGATGCACCCGAACAGAGT
LBP-BPI	mussel_c39362	GCTTCACTGATACTGCTTGCCC	CCACGGTGGAGCAGCATGGA
Immune lectin receptor 2	mussel_rep_c70917	TGGACACTGCTACCATTATGGGACC	CGATTGGTCATAGCTCCAACGCC
Signaling genes			
Toll-like receptor 2	mussel_c2881	CCAGGAGGACTCGGATGACACA	ACTCCGGAACTTGGAGAGCACG
TRAF6	mussel_c9675	CACCTATTTCCGCTTCCCGCC	TGGAGGGTGGTGGTGCTCTT
MyD88	mussel_c3721	TCTGCCACACCCAACAACGC	TCGAGACTGAGGTTCTCGCACA
EGF	mussel_c3243	GGGACACATTGCGAAACGGC	TTCGCCCCGTAAATCCAGGCA
Transcription genes			
STAT-SH2	mussel_c5862	AGCTGAAACAGGGCGTGGTC	GACAAATCCAGCCACATGCCCA
Jun-like	mussel_c14202	CGCCAACACCGACACAGTTCA	AACCCCCGGGGAGTGTTGTT
Effector genes			
Lysozyme	mussel_c15166	GCTGTATCTGTCAGGTTGAAATCGC	TGGTCCTCCGTTATGGATGCTGGC
Glutathione peroxidase I	mussel_c23951	TTAACGGCGTCGTCGCTTGG	TGGCTTCTCTCTGAGGAACAACTG
Metallothionein	mussel_c72489	TCGGCACTGTCCACACAAAACC	CAACCGGAAGCGGATGTGGC
Cytolysin	mussel_lrc36522	CGGTTGCTGTGTAGCCGCAT	TTGGCGTCCAGAGACCGGAG

added as suspensions, to 2 L mussel containing beakers. Experimental infections were kept at 7–8 °C in the LabHorta aquarium systems. Four animals from each experimental infection beaker were dissected at 12 h post-infection time and remaining four mussels dissected afterwards at 24 h post-infection time.

2.3 Total RNA extraction

Total RNA was extracted from gill tissues with TriReagent® (Ambion) and further purified with the RiboPure™ Kit (Ambion) following the manufacturer's specifications and re-suspended in nuclease-free, DEPC-treated water. Total RNA quality preparations and concentrations were assessed by the $A_{260/280}$ and $A_{260/230}$ spectrophotometric ratios using the NanoVue spectrophotometer (General Electric, Healthcare Life Sciences. The cDNA was synthesized with SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions, using 5 µg total RNA per sample. Equal amounts of total RNA were used in all cDNA syntheses. The cDNA concentration was measured using the NanoVue spectrophotometer as above.

2.4 Gene expression analyses

Gene expression analyses from 12 h and 24 h post-infection gill samples were carried out using a mixture of four gill samples by means of quantitative PCR (qPCR) following the MIQE guidelines (Bustin et al., 2009). The immune genes selected in this study (Table 1), were Rhamnose-binding lectin, Peptidoglycan recognition protein (PGRP), Serpin, Aggrecan, Bactericidal/permeability-increasing protein-Lipopolysaccharide-binding protein (BPI-LBP), Immune lectin receptor 2, Toll-like receptor 2, Tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), Myeloid differentiation primary response gene 88 (MyD88), Epidermal growth factor (EGF), STAT-SH2, Jun-like, Lysozyme, Glutathione peroxidase I, Metallothionein and Cytolysin gene. qPCR assays were performed with the CFX96™ Real-Time (Bio-Rad) using the same amount of cDNA concentration together with 10 µL of SYBR®green (Fermentas), 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer and nuclease-free water in a final volume of 20 µL per reaction.

The standard cycle conditions used in this study were 95 °C for 10 min, 94 °C for 20 s, 52 °C for 20 s and 68 °C for

Table 2. Efficiency values for target genes according to the immune functional categories, Recognition, Signaling, Transcription and Effector genes.

Target genes	Efficiency values
Housekeeping gene	
28S	−3.16
Recognition genes	
Rhamnose binding lectin	−3.25
PGRP	−3.4
Serpin	−3.91
Aggrecan	−3.78
LBP-BPI	−3.2
Immune lectin receptor 2	−3.11
Signaling genes	
Toll-like receptor 2	−3.44
TRAF6	−3.43
MyD88	−3.6
EGF	−3.67
Transcription genes	
STAT-SH2	−3.75
Jun-like	−3.42
Effector genes	
Lysozyme	−3.13
Glutathione peroxidase I	−2.74
Metallothionein	−3.33
Cytolysin	−3.21

30 s followed by 40 cycles of 65 °C for 5 min and 95 °C for 5 min. Gene expressions quantifications were normalised using the 28S ribosomal RNA gene as the housekeeping gene. Data analyses were based on the $\Delta\Delta C_t$ method with normalisation of the raw data to the housekeeping gene expression values. The forward and reverse sequences used in qPCR are shown in Table 1. Two technical replicates were obtained from qPCR and data were expressed as means and Standard Deviation.

The immune genes analysed in the present study, were categorised into four groups of functional genes referred as Recognition, Signaling, Transcription and Effector genes (Bettencourt et al., 2010). The primer pair efficiency (Table 2) was analysed in consecutive dilutions of cDNA through the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA (Livak and Schmittgen, 2001).

2.5 Statistical analyses

The statistical analyses were performed with the software package IBM SPSS Statistic 19 and R software. The gene expression data were expressed as Mean ± Standard Deviation (SD). The differences in gene expression, bacterial exposure effect and the duration of experimental infections (12 h and 24 h post-infection) were evaluated using analysis of variance; where the assumption of normality and homogeneity of variances (Leven's test) was not met, non-parametric tests such as Mann-Whitney's were used instead. Significance levels for tests were 5 %. These analyses together with the post-hoc Tukey HSD test were done with the SPSS Statistic 19 software. Multivariate analysis was conducted with R software, and cluster analysis performed on the euclidian distance matrix using the UPGMA (Unweighted Pair-group Method using Arithmetic Average) method. Results from qPCR were subjected to Hierarchical clustering dendrograms using UPGMA method and taking into account, on the one hand, the experimental infections clustering (*Flavobacterium*, Pool, *V. anguillarum*, *V. splendidus*, *V. alginolyticus* and Control), established for 12 h (Fig. 2) and on the other hand, same experimental infections clustering for 24 h (Fig. 3) post-infection times. This method was also applied for gene cluster dendrograms set for 12 h (Fig. 4) and 24 h (Fig. 5) post-infection times. In addition, Heatmaps were produced and showed (Figs. 6 and 7). The Penalty function of KGS (Grum and Atieno, 2007) was used to find the appropriate number of clusters.

2.6 SDS-PAGE

Gills from *B. azoricus* animals infected with the marine bacterial pathogens, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, a pool of these Vibrios, and *Flavobacterium* were used for protein assessment and separation in polyacrylamide gel electrophoresis experiments. Control gill samples from animals bathing in seawater were also analysed. SDS-PAGE analyses were carried out with homogenates consisting of four gills mixtures corresponding to 12 h and 24 h post infection times. Gill samples were prepared with 2 mL of a 45 mL solution consisting of 500 µL 0.1 M DTT, 500 µL Protease Arrest™ 100x (Calbiochem®), 500 µL EDTA 0.5 M 100×, 200 µL Triton® X-100, 500 µL SDS 10 % and Tris-HCL 6.8 pH. Homogenates were centrifuged at 4000 rpm for 15 min, at 4 °C, followed by a second centrifugation at 13 200 rpm for 30 min, at 4 °C. Afterwards, to the supernatants equal volume of 1X (Applichem A3484) loading buffer was added.

The samples were denatured at 95 °C, for 10 min, in a water bath and subsequently, centrifuged at 13 200 rpm for 30 min. The supernatant was then loaded onto pre-cast 4–12 % Bis-Tris NuPAGE® Novex polyacrylamide gels (Invitrogen) and ran with 1X NuPAGE® MES running buffer at 90 V for 1 h.

A reference protein (around 28 KDa) was visually examined, in all samples, to be used as a protein loading control reference in 12 h and 24 h post-infection protein samples. 5 µL protein ladder (SeeBlue® Pre-Stained Standard Invitrogen) were loaded separately for protein molecular mass

determination. Coomassie blue solution (Phast Gel TM Blue Tablet R, GE Healthcare) was used for Gel staining, during 1 h, at 45 °C with gentle agitation, following the manufacturer's instructions. Gels were rinsed twice with destain solution (70 % distilled water, 20 % methanol and 10 % acetic acid glacial), under gentle agitation, for 1 h, at 45 °C until protein bands were adequately visualised.

3 Results

In vivo incubation experiments carried out with different live *Vibrio* strains and *Flavobacterium* induced differential gene expression at 12 h and 24 h post-infection times. Among the functional category "recognition genes" tested, the Immune lectin receptor 2 revealed the highest expression level, at 12 h infection, in the presence of *Flavobacterium* whereas incubations with *V. alginolyticus* and the pool of Vibrios resulted in a slightly above seawater-incubation control expression level for this immune gene (Fig. 1a). Similarly, the Serpin gene was induced to its highest level by *Flavobacterium* and to a lesser extent by *V. anguillarum* (Fig. 1a). Gene expression results obtained for the Rhamnose-binding lectin, PGRP, Aggrecan, LPB-BPI were below seawater-incubation gene expression levels suggesting that *B. azoricus* gill tissues retain an immune discriminatory capacity while inducing differential transcript expressions, relative to the microorganism tested in comparison to seawater expression levels. In some cases, up-regulation was seen for the Immune lectin receptor 2 in the presence of *Flavobacterium* whereas down-regulation was observed for the same gene in the presence of *V. splendidus* and *V. anguillarum* (Fig. 1a). The 24 h post-infection transcriptional activity was significantly different from the transcriptional activity at 12 h post-infection time (Mann-Whitney test Sig = 0). At 24 h, the Immune lectin receptor 2 gene presented again the highest level of expression among the immune recognition genes tested, in the presence of *V. splendidus*, in contrast to what was observed for *Flavobacterium* at 12 h. The Immune lectin receptor 2 up-regulation is still noticeable for *V. anguillarum* and the pool of Vibrios, in divergence to the effect seen with *V. alginolyticus* whether at 12 h or 24 h post-infection (Fig. 1b).

Regarding the functional category "signaling genes", *B. azoricus* infected with *V. alginolyticus* revealed the highest expression level for Toll-like receptor 2 (TLR2) at 12 h time point, followed by *Flavobacterium* and to a lesser extent by *V. splendidus* (Fig. 1c). In the same way, MyD88 gene was induced to its highest level by *Flavobacterium* and also by *V. alginolyticus* compared to the control seawater-incubation animals. On the contrary, down-regulation was observed for the same gene in the presence of *V. splendidus*, the pool of Vibrios and *V. anguillarum* (Fig. 1c). The 24 h post-infection transcriptional activity was different to that of the 12 h infection time. The TLR2 gene presented again the highest level of expression among the signaling genes tested, in the presence of *V. splendidus* at 24 h in contrast to gene expressions values obtained with *V. alginolyticus* at 12 h. Below-seawater control gene expression levels were found for TRAF6, MyD88 and EGF. Moreover, TLR2 was up-regulated in the presence of *V. splendidus* and the pool of Vibrios at 24 h (Fig. 1d).

As for the category "transcription genes", Jun-like gene revealed the highest expression level at 12 h infection, in the presence of *Flavobacterium* as well as for the pool of Vibrios (Fig. 1e). On the other hand, infections carried out with *V. splendidus, V. alginolyticus and V. anguillarum* showed reduced expression for STAT-SH2 gene and Jun-like gene at 12 h infection (Fig. 1e). The 24 h post-infection transcriptional activity revealed some differences, as for the STAT-SH2 gene and Jun-like which presented an increase level of expression in the presence of *V. splendidus* at 24 h, as opposed to what was observed at 12 h. At 24 h, Jun-like gene revealed the highest expression level in *V. splendidus* infections. Below seawater-incubation gene expression levels were found in the presence of *V. alginolyticus*, *V. anguillarum*, pool of Vibrios and *Flavobacterium* at 24 h (Fig. 1f).

Within the immune category "effector genes", Lysozyme showed the highest expression level, in 12 h *Flavobacterium* infections (Fig. 1g). However, Metallothionein and Glutathione peroxidase I revealed some expression compared to the control seawater-incubation group of animals, in the presence of the same bacterium, at 12 h (Fig. 1g). Moreover, the effector genes showed down-regulation in the presence of *V. splendidus*, *V. alginolyticus*, *V. anguillarum* and the pool of Vibrios (Fig. 1g). The 24 h post-infection transcriptional activity for cytolisin revealed a higher expression level compared to 12 h (Fig. 1h).

The statistical analyses applied to our experimental conditions, evidenced significant differences ($p < 0{,}05$) within the expression levels at 12 h and 24 h post infection, and also between levels of gene expression. These analyses include Mann-Whitney test (Sig = 0) and ANOVA with post-hoc Tukey HSD test. At 12 h infection time, 69 % of the genes were up-regulated whereas 31 % were down-regulated. At 24 h infection time, 37.5 % of the genes were up-regulated whereas 62.5 % were down-regulated genes. When comparing the number of genes induced by bacteria, at 12 h, *V. splendidus* induced 5 % of the total genes, *V. alginolyticus* induces 17 %, *V. anguillarum* 5 %, the pool of Vibrios 17 % and *Flavobacterium* 56 % of the total genes. At 24 h, *V. splendidus* induced 50 % of the total genes, *V. alginolyticus* induced 0 %, *V. anguillarum* 10 %, the pool of Vibrios 30 % and *Flavobacterium* 10 % of the total genes (Fig. 8).

Our descriptive data analyses were followed by multivariate statistical analyses to better illustrate how vent mussels respond to bacterial infections while up-regulating and down-regulating immune genes during bacterial infections and allow for exploratory analysis to see how the gene expression results group together based on similarity of features, and thus testing the premise that their innate immune system is capable of discriminating different *Vibrio* strains.

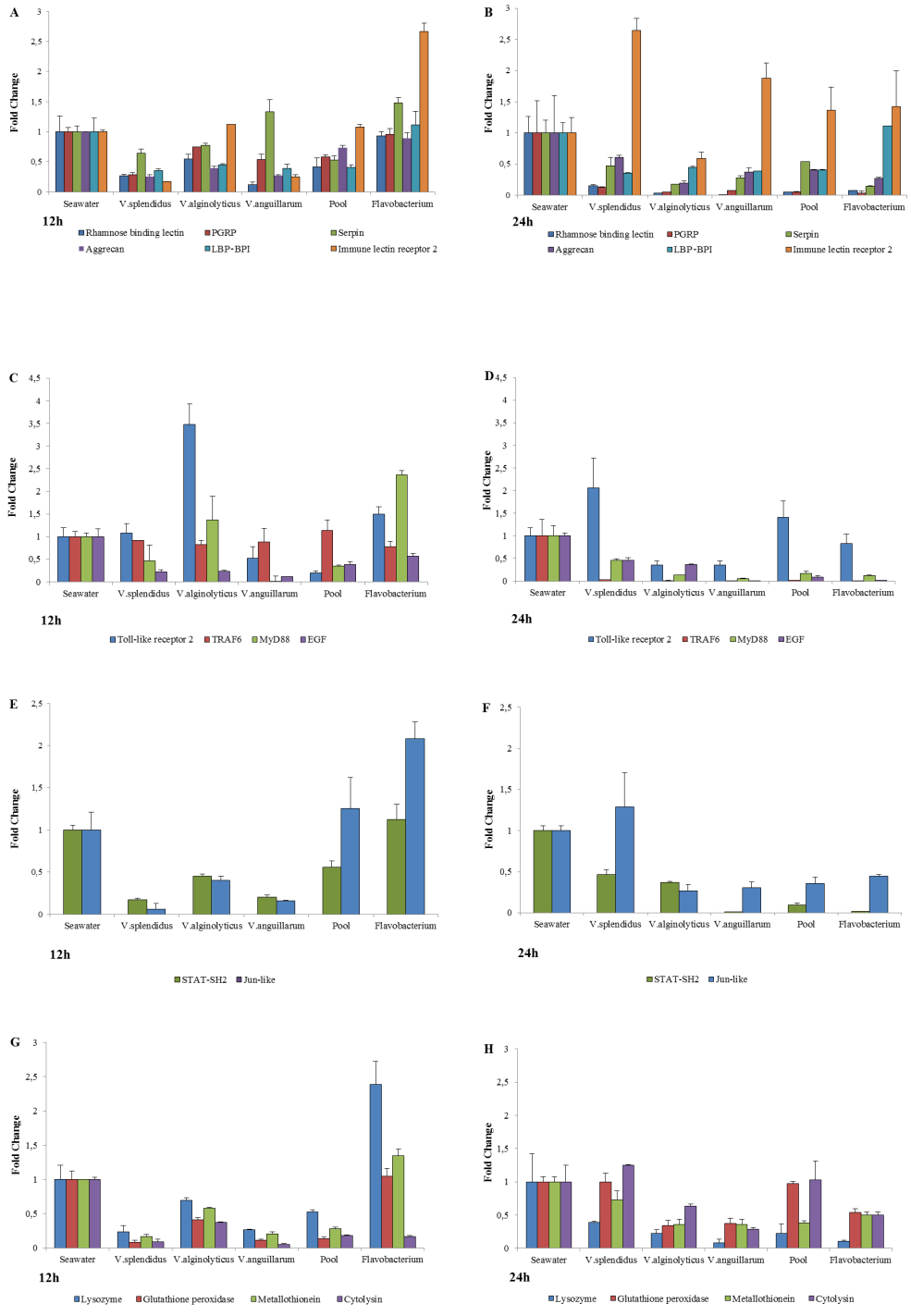

Fig. 1. Differential expression of immune genes. **(A)** Expression levels of immune recognition genes (Rhamnose-binding lectin, PGRP, Serpin, Aggrecan, LBP-BPI and Immune lectin receptor 2) from *B. azoricus* infected gills at 12 h infection time. **(B)** Expression levels of immune recognition genes as in **(A)**, from *B. azoricus* infected gills at 24 h infection time. **(C)** Expression levels of immune signaling genes (Toll-like receptor 2, TRAF6, MyD88 and EGF) from *B. azoricus* infected gills at 12 h infection time. **(D)** Expression levels of immune signaling genes as in **(C)**, at 24 h infection time. **(E)** Expression levels of immune transcription genes (STAT-SH2 and Jun-like) from *B. azoricus* infected gills at 12 h infection time. **(F)** Expression levels of immune transcription genes as in **(E)**, at 24 h infection time. **(G)** Expression levels of immune effector genes (Lysozyme, Glutathione peroxidase, Metallothionein and Cytolysin) from *B. azoricus* infected gills at 12 h infection time. **(H)** Expression levels of immune effector genes as in **(G)**, at 24 h infection time. Data expressed as Means and Standard Deviation with two technique replicates. Bars represent the level expression (Fold change) of target gene, in different experimental conditions, and normalised to the housekeeping gene 28S.

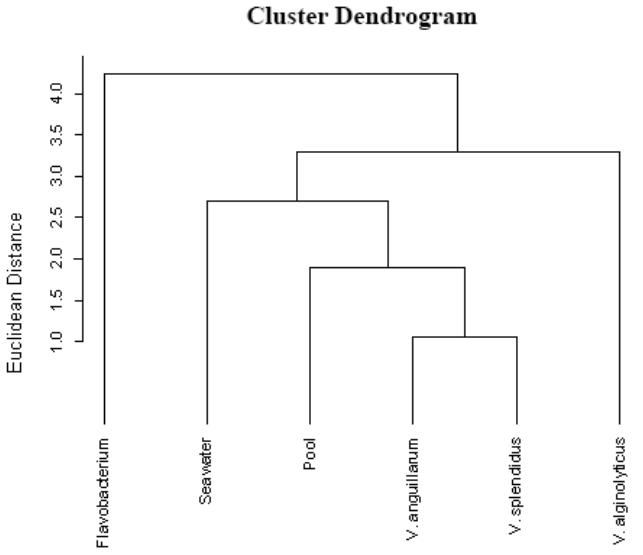

Fig. 2. Hierarchical clustering Dendrogram of Experimental condition (*Flavobacterium*, pool of Vibrios, *V. anguillarum*, *V. splendidus*, *V. alginolyticus*) and Control (Seawater) of mussel *B. azoricus* at 12 h post-infection through Euclidean distance, using UPGMA method.

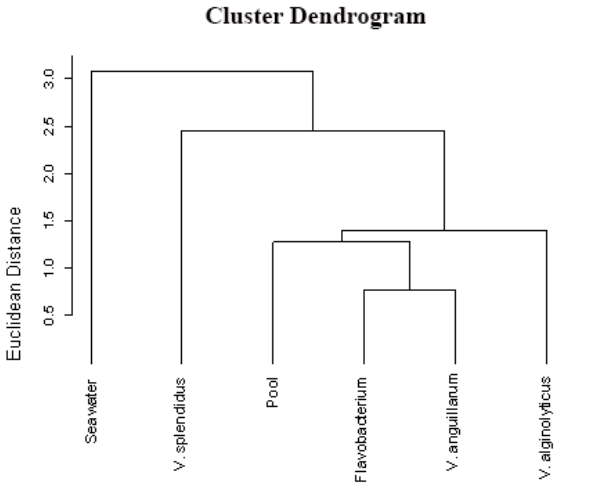

Fig. 3. Hierarchical clustering Dendrogram of Experimental condition (*Flavobacterium*, pool of Vibrios, *V. anguillarum*, *V. splendidus*, *V. alginolyticus*) and Control (Seawater) of mussel *B. azoricus* at 24 h post-infection through Euclidean distance, using UPGMA method.

The hierarchical clustering dendrogram demonstrated how data originated from bacterial infections and from control-seawater conditions were clustered together with the quantitative gene expression pattern they determined. Four cluster dendrograms were considered with the analysis of bacterial infection conditions results obtained at 12 h incubation time (Fig. 2). The first cluster was formed with *Flavobacterium* which caused distinct responses in *B. azoricus*. The second cluster included seawater-incubation samples, regarded as our experimental control. The third cluster was formed with the pool of Vibrios, *V. anguillarum* and *V. splendidus* and the fourth cluster was formed with *V. alginolyticus* infection. This is in agreement with the levels of gene expressions for Immune lectin receptor 2 and Toll-like receptor 2 (Fig. 1a and b).

As for the cluster dendrogram results obtained for 24 h incubation time (Fig. 3), three clusters were defined, which in this case included a cluster consisting of the control seawater-incubation samples, a second consisting of *V. splendidus*, and a third cluster formed upon the remaining *Vibrio*, the pool of Vibrios and *Flavobacterium* results This correlates with *V. splendidus* infection, that results, in this study, in up-regulated immune genes (Fig. 1b, d, f and h).

The multivariate statistical analyses also provided a representation on how signaling pathways may be selectively activated in *B. azoricus* during immune responses triggered by marine bacteria over the course of infection. Clustering gene expression data using hierarchical clustering R analysis, relative to 12 h (Fig. 4) and 24 h post-infection (Fig. 5), gave rise to a gene cluster dendrogram where fours clusters were

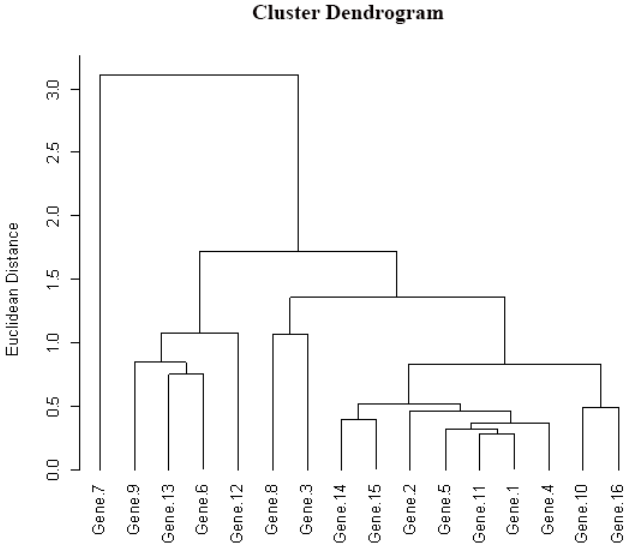

Fig. 4. Gene Cluster Dendrogram of mussel *B. azoricus* at 12 h post-infection through Euclidean distance, using UPGMA method. The numeration of Genes represent: (1) Rhamnose binding lectin; (2) PGRP; (3) Serpin; (4) Aggrecan; (5) LBP-BPI; (6) Immune lectin receptor 2; (7) Toll-like receptor 2; (8) TRAF6; (9) MyD88; (10) EGF; (11) STAT-SH2; (12) Jun-like; (13) Lysozyme; (14) Glutathione peroxidase I; (15) Metallothionein and (16) Cytolysin.

evidenced. In Fig. 4 (12 h) a first cluster was constituted only by the Toll-like receptor 2 (TLR2) gene. The fact that TLR2 is included as in one single cluster is coincident with its distinctiveness in our gene expression studies. It represents the most inducible of the signaling genes for both 12 h and 24 h infections (Fig. 1c and d). A second cluster was formed with

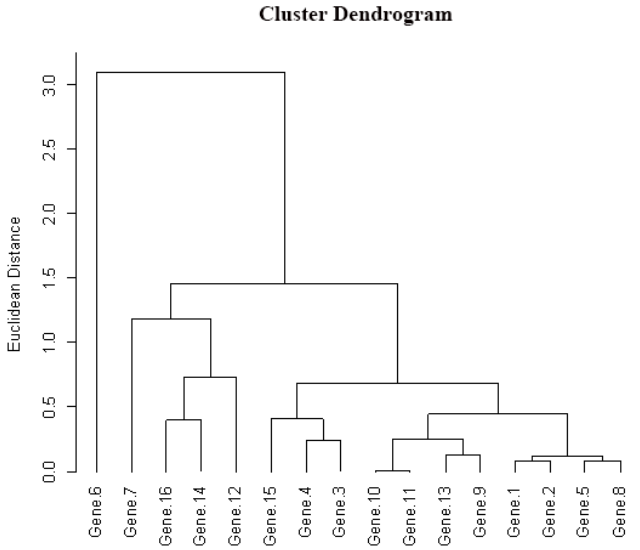

Fig. 5. Gene Cluster Dendrogram of mussel *B. azoricus* at 24 h post-infection through Euclidean distance, using UPGMA method. The numeration of Genes represent: (1) Rhamnose binding lectin; (2) PGRP; (3) Serpin; (4) Aggrecan; (5) LBP-BPI; (6) Immune lectin receptor 2; (7) Toll-like receptor 2; (8) TRAF6; (9) MyD88; (10) EGF; (11) STAT-SH2; (12) Jun-like; (13) Lysozyme; (14) Glutathione peroxidase I; (15) Metallothionein and (16) Cytolysin.

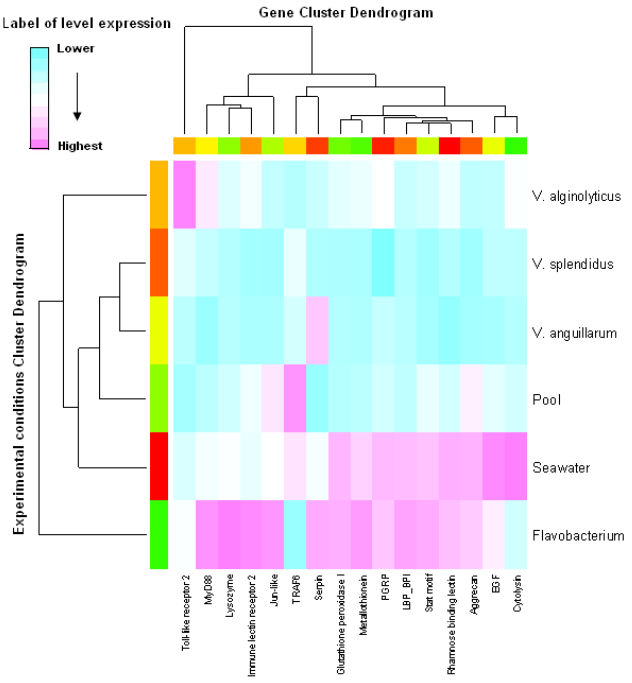

Fig. 6. Hierarchical Clustering Heatmap Plot of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The heatmap represents a grid of coloured points where each colour represents a level of gene expression (Fold change). The rows represent Experimental conditions (*V. alginolyticus*, *V. splendidus*, *V. anguillarum*, pool of Vibrios, Seawater and *Flavobacterium*) and the columns represent 16 genes analysed at 12 h of infections. The grid coordinates correspond to the sample by gene combinations where the dark pink colour corresponds the highest expression and dark blue corresponds to the lowest expression.

MyD88, Lysozyme, Immune lectin receptor 2 and Jun-like genes. A third cluster included TRAF6 and Serpin gene, both genes, putatively involved in the Toll signaling pathway. A fourth cluster was formed with Glutathione peroxidase, Metallothionein, PGRP, LBP-BPI, STAT-SH2, Rhamnose binding lectin, Aggrecan, EGF and Cytolysin genes (Fig. 4).

The genes cluster dendrogram regarding expression levels obtained at 24 h incubation time (Fig. 5), indicated that four clusters were obtained. The first cluster was formed with Immune lectin receptor 2 and second cluster by TLR2. The third cluster was formed with Cytolysin, Glutathione peroxidase and Jun-like. The remaining genes were grouped in a fourth cluster (Fig. 5).

Our statistical analyses also included Heatmaps to illustrate how differential gene expression data group together based on similarities of features between the experimental conditions, control seawater, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, the pool of Vibrios and *Flavobacterium* incubations data and the expression of sixteen immune genes at 12 h (Fig. 6) and 24 h post-infection times (Fig. 7).

As for the gene cluster dendrograms, the TLR2 gene was expressed to its highest level upon *V. alginolyticus* infection which is visualised in the Heatmap as a dark pink colour whereas for other *Vibrio* infections, lower levels of expression are represented by other colours. The lowest level of expression is represented by a dark blue colour such as in the case of pool of Vibrios infection. The Heatmap corresponding to 24 h post-infection results (Fig. 7), indicates the down-regulation of the Metallothionein, Serpin, EGF, STAT-SH2, Lysozyme, MyD88, Rhamnose-binding protein, PGRP, LBP-BPI and TRAF6 genes, whereas Glutathione peroxidase I showed a similar level of expression as the control seawater, *V. splendidus* and the pool of Vibrios incubations.

The SDS-PAGE protein analyses revealed some differences between *B. azoricus* gill samples from the 12 h to 24 h infection experiments (Fig. 9). A reference protein band corresponding to 28 KDa was used as a protein loading control in 1-D comparative protein electrophoresis using samples from 12 h and 24 h Vibrios and *Flavobacterium* infections. This reference protein band (Appendix A) was subsequently analysed by mass spectrometry followed by Mascot searches based on probability Mowse Score revealing homologies with a *Branchiostoma floridae* hypothetical protein (score 119 with 99 % homology) and to actin proteins by searches in the NCBI database (Table 3, Appendix A). The rectangles highlight the reduced amount of bands from 12 h to 24 h and the white bracket suggests an increase of the bands at 12 h infection (Fig. 9).

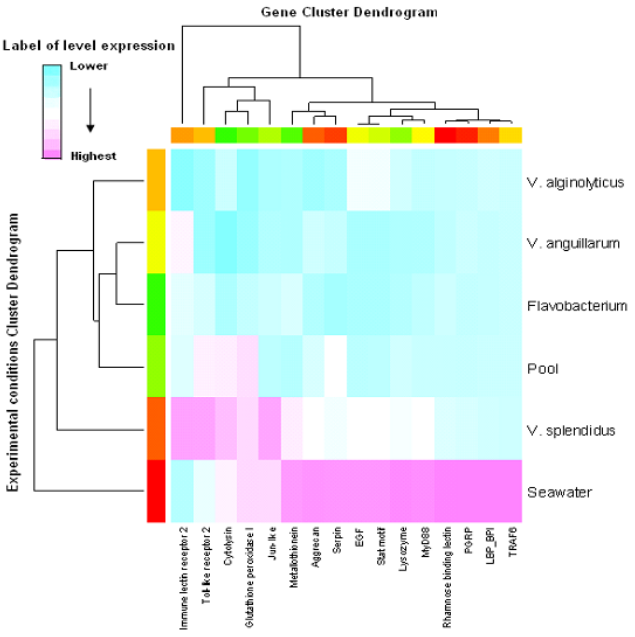

Fig. 7. Hierarchical Clustering Heatmap Plot of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The heatmap represents a grid of coloured points where each colour represents a level of gene expression (Fold change). The rows represent Experimental conditions (*V. alginolyticus*, *V. splendidus*, *V. anguillarum*, pool of Vibrios, Seawater and *Flavobacterium*) and the columns represent 16 genes analysed at 24 h of infections. The grid coordinates correspond to the sample by gene combinations where the dark pink colour corresponds the highest expression and dark blue corresponds to the lowest expression.

SDS-PAGE comparison protein profiles using samples from 12 h and 24 h infection time, revealed differences around 18 kDa regardless of the bacteria used. Within this electrophoretic mobility area, the occurrence of inducible bands was visible for 24 h samples whereas in the 12 h samples these were still not evidenced (Fig. 9).

4 Discussion

Understanding the molecular mechanisms underlying defense reactions and the activation of genes involved in signaling pathways leading to innate immune responses, may provide helpful information, on how animals respond to environmental microorganisms that in the case of vent mussels may involve interaction with potential pathogens from the surrounding vent environment. The present studies presented a first insight into how vent mussels may respond to the presence of distinct marine bacteria bringing evidence for immune discriminatory capabilities not seen in previous studies conducted with deep-sea vent organisms. We have addressed the problem of microbial threat and the need for immunity that must exist in the deep-sea mussels as for their congeners living in estuarine environments, them too, subjected to microbial threat while focusing our gene expression analyses on "canonical" immune genes already evidenced in our and others previous studies, more so, when new sequencing techniques were made available such as next generation sequencing methods, high-throughput deep sequencing, applied to *de novo* transcriptome sequencing (454 and illumina). Our transcriptome sequencing, published in 2010, provided a source of data for the discovery and identification of new genes of which several putative immune genes were revealed and their physical nature put in evidence by means of qPCR (Bettencourt et al., 2010).

Our results suggest that *B. azoricus* is equipped with discriminatory capabilities in that it enables distinct responses to marine bacterial pathogens such as *V. splendidus*, *V. alginolyticus*, *V. anguillarum* and *Flavobacterium*. The gene targets tested showed differences in expression over infection time and nature of the bacterium used and thus pointing at a dual modulation of gene expression and immune discriminatory capabilities of the host vent mussel. The TLR2 expression profiles are in agreement with a role in the recognition of different bacterial infectious strains while expressing distinctively according to the *Vibrio* strain and time of infection. TLR2 presented distinct expression levels at 12 h and 24 h of infection as demonstrated by its down-regulation upon *V. anguillarum* incubation and up-regulation upon *V. alginolyticus* within the same 12 h post-infection time (Fig. 1c). This suggested that pathogens activate different transcriptional activities of genes involved in signaling pathways such as the TLR2. The TLR family includes receptors found both at the intracellular level and cell surface triggering the induction of cytokines essential for the innate immune response following the recognition of microbial products and foreign nucleic acids, the signature of invading viruses and certain bacteria, sensed intracellularly (Blasius and Beutler, 2010). The fact that TLR2 is included as in one single hierarchical dendrogram cluster is coincident with its singularity during our gene expression studies. It represents the most inducible of the signaling genes for both 12 h and 24 h infections (Fig. 1c and d). MyD88 is another signaling molecule that is involved in the intracellular homeostasis, through phagocytosis and the synthesis of microbial effector molecules (Sancho and Sousa, 2012) such as lysozyme and thus represents a key protein in the innate immune responses of invertebrates against bacterial infections (Zhao et al., 2010). This is corroborated by gene expression results for Immune lectin receptor 2, MyD88 and lysozyme which were up-regulated in mussels infected with *Flavobacterium* at 12 h suggesting that *B. azoricus* was under high immune transcriptional activity due to infection since these genes are involved in opposing and eliminating pathogenic microorganisms.

Lectins constitute a group of sugar-binding proteins that recognise specific carbohydrate structures allowing agglutination of foreign cells and the stimulation of phagocytosis (Endo et al., 2010; Kilpatrick, 2002) assuming a crucial role

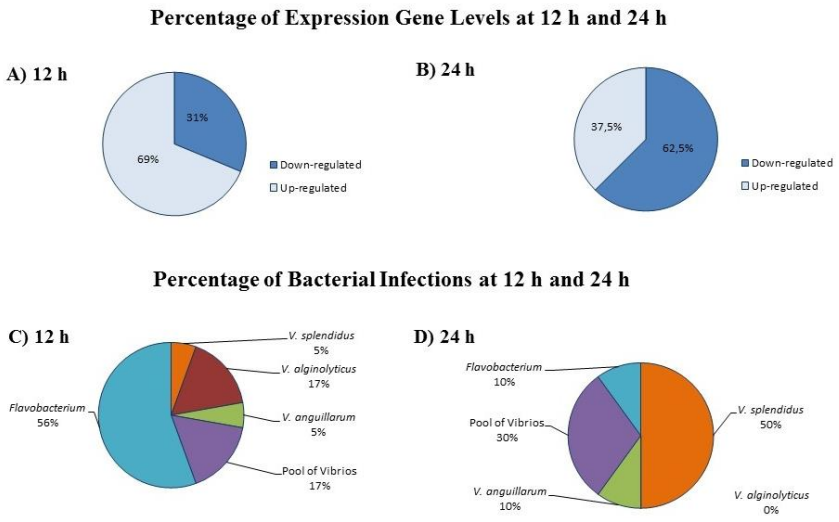

Fig. 8. Percentage of gene expression levels and percentage of genes induced by bacterial infections at 12 h and 24 h infection time based on qPCR results. **(A)** and **(B)** Percentage of down- and up-regulated genes at 12 h and 24 h respectively. **(C)** and **(D)** Percentage of genes induced by bacterial infections (*V. splendidus*, *V. alginolyticus*, *V. anguillarum*, pool of Vibrios and *Flavobacterium*) based on percentage of up-regulated genes in *B. azoricus.*

Table 3. Mascot search results of *B. azoricus* peptides that match with sequence of Accession Nr., Mass, Score and Description that was based on probability Mowse Score.

Accession Nr.	Mass	Score	Description
gi|260794036	41 966	119	hypothetical protein BRAFLDRAFT_114535 [*Branchiostoma floridae*]
gi|344250136	27 440	111	Actin, cytoplasmic 2 [*Cricetulus griseus*]
gi|169643685	39 426	110	beta actin isoform 1 [*Solea senegalensis*]
gi|4376057	420 709	110	actin [*Oreochromis mossambicus*]
gi|3182897	41 971	109	RecName: Full = Actin, cytoplasmic; Contains: RecName: Full = Actin, cytoplasmic, N-terminally processed
gi|229472802	41 993	109	actin [*Crassostrea gigas*]
gi|118419975	40 937	108	beta-actin [*Megaptera novaeangliae*]
gi|238866623	12 076	106	beta-actin [*Menidia beryllina*]
gi|298155653	922 284	105	actin [*Nectria cyanostoma*]
gi|84314104	22 492	105	actin [*Trichoderma ovalisporum*]

in innate immunity of bivalves (Bulgakov et al., 2004). However, the Rhamnose-binding lectin and the Immune lectin receptor 2, two lectin-like immune molecules whose expression profiles differed distinctively between 12 h and 24 h infection time, seem to suggest that infections carried out with *Vibrio* are yet to induce Rhamnose-binding lectin transcript levels above seawater-incubation experiments (Fig. 1a and b), whereas this transcript was markedly higher for both 12 h and 24 h *Flavobacterium* incubations. In contrast, 12 h and 24 h infections with *Flavobacterium* induced higher levels of immune lectin receptor 2 transcripts, above the control seawater-incubation expression experiments.

In a recent transcriptome profiling study, using the Pacific *Crassostrea gigas* as a model to address immune responses to the virulent *Vibrio splendidus* and *Vibrio aestuarianus* bacteria, several immune genes were characterised in relation to the oyster capability to survive pathogenic *Vibrio* infections, including the Rhamnose-binding lectin, C-type lectin 2 and Metallothionein IV whose expression were up-regulated in surviving oysters (De Lorgeril et al., 2011). Xue et al. (2010) also evidenced the involvement of a new lysozyme in the oyster *Crassostrea virginica* which also agrees with the finding that *B. azoricus* lysozyme is up-regulated in *Flavobacterium* infections. Zhao et al. (2010) demonstrated that the lysozyme mRNA transcript from *Venerupis philippinarum* clam is most abundantly expressed in gills tissues and hemocytes. This lysozyme is similar to molluscan lysozymes. qPCR analyses using clams infected with *V. anguillarum*, showed that the lysozyme mRNA levels in hemocyte were down-regulated abruptly from 6 h to 12 h post-infection and increased to a peak value at 72 h. The qPCR results using gills from mussel infected with *V. alginolyticus*, *V. anguillarum*, pool of Vibrios and *Flavobacterium* revealed a decrease of lysozyme expression levels from 12 h to 24 h (Fig. 1g and h).

The multivariate statistical analyses also hinted at how signaling pathways may be activated in *B. azoricus* during

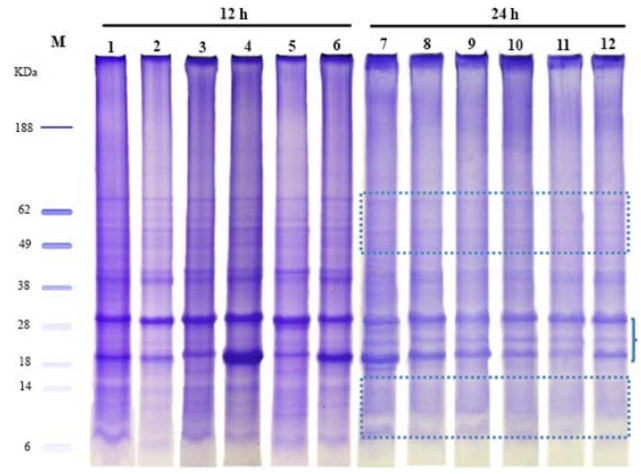

Fig. 9. SDS-PAGE of mix gills protein samples from *B. azoricus*, incubated in the presence of Seawater, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, pool of Vibrio and *Flavobacterium*. The lanes 1, 2, 3, 4, 5 and 6 represent samples at 12 h infection and the lanes 7, 8, 9, 10, 11 and 12 correspond to 24 h infection samples. The rectangles boxes and braces indicate the main differences in protein patterns (18 kDa) between 12 h and 24 h of infection. Molecular mass marker (M, SeeBlue®) is indicated.

immune responses triggered by marine bacteria. The cluster analysis applied to gene expression data was addressed in this study to arrange genes according to similarity in pattern of gene expression. The results were displayed graphically, presenting the clustering and the underlying expression data simultaneously in a form that would help us explore how signaling pathways may be activated in *B. azoricus* during immune responses triggered by marine bacteria. Clustering data using hierarchical clustering R analysis, regarding 12 h (Fig. 4) and 24 h post-infection (Fig. 5) gene expression levels, proven to be resourceful in the planning of future experiments involving immune gene expressions that may now be selected on the basis of their relativeness and similarity in pattern of gene expression as evidenced in our analyses by the establishment of distinct cluster groups. The hierarchical clustering approach as dendrograms and/or Heatmaps provided clear examples in our study of such clustering making that included Aggrecan, STAT-SH2 and EGF within the same cluster. Aggrecan is a proteoglycan of the extracellular matrix that can regulate the permeability membrane and is consequently involved in host defense and wound repair (Velleman, 2000; Esko et al., 2009). The STAT gene is involved in mediating functions and is associated with innate immunity (Ihle, 2001), while epidermal growth factor gene (EGF) is associated with proliferation, differentiation of epidermal cells (Tanabe et al., 2008). EGF is activated by signal transducers STAT-SH2, and both were down-regulated in relation to control level expression in the present study. Our results suggested high proximity or relativeness between EGF and STAT-SH2 which are likely linked in cellular processes (Fig. 5).

The genes dendrogram and Heatmap plots support the premise that genes were group according to their function in immune signaling pathways. The gene expression profiles changed according to the bacterial pathogen tested, and the time period of experimental infections. These conditions were statistically confirmed ($p < 0.05$) with Mann-Whitney test and Tukey HSD test. Therefore, it is assumed from this study that *B. azoricus* mussels were under immune challenging conditions, since comparative results from the control seawater-incubations and Vibrio infections were significantly different.

5 Conclusions

This study demonstrates that vent mussels display diverse gene expression profiles that in general decreased from 12 h to 24 h time of infections. The immune gene responses were modulated at two levels that are, over the course of time and according to the bacteria tested. Infections carried out with *Flavobacterium*, induced the highest gene expression (56 % of the total genes induced) levels at 12 h infection time, whereas *V. splendidus* infections revealed greater gene expression levels at 24 h infection time (50 % of the total genes induced). Also the SDS-PAGE analyses, pointed at differences between 12 h and 24 h infection times which could be interpreted as a result of an elevated transcriptional status in animals exposed to *Vibrio* for 24 h that in turn ensued in the synthesis of more proteins. Most of the immune genes used in this experimental study seemed to be generally down-regulated upon *Vibrio* and *Flavobacterium* infection, a phenomenon that we relate to specific dynamics, between microorganisms from the extracellular milieu and *B. azoricus* gill epithelial cells that comprise specific immune responses and the recruitment of distinct signaling pathways as well as the presence of endosymbiont bacteria.

Taken together, we analysed the effect of bacterial infections in our mussel species while focusing our experiments on infections conducted at atmospheric pressure and not simulating "natural environment" found at hydrothermal vents. In this way, our deep-sea vent mussel became a "shallow-water" animal model while keeping its deep-sea vent genomic "make-up" and transcriptional capabilities (responsiveness), in our land-based aquarium system, without the characteristic hydrostatic pressure levels found at the deep-sea vent environments. Our results concur with other's published work, in the field of innate immunity, combining high-throughput and transcriptome sequencing analyses with experimental data obtained by means of quantitative PCR and pointing at the up-regulation of canonical genes involved in innate immunity.

Appendix A

***B. azoricus* Protein Sequence**

1	MCDEDVAALV VDNGSGMCKA GFAGDDAPRA VFPSIVGRPR HQGVMVGMGQ
51	KDSYVGDEAQ SKRGILTLKY PIEHGIVTNW DDMEKIWHHT FYNELRVAPE
101	EHPVLLTEAP LNPKANREKM TQIMFETFNS PAMYVAIQAV LSLYASGRTT
151	GIVLDSGDGV SHTVPIYEGY ALPHAIIRLD LAGRDLTDYQ MKILTERGYS
201	FTTTAEREIV RDIKEKLCYV ALDFEQEMQT AASSSSLEKS YELPDGQVIT
251	IGNERFRAPE ALFQPSFLGM ESAGVHETTF NSIGKCDIDI RKDLYANTVL
301	SGGTTMFPGI ADRMQKEISA LAPPTMKIKI IAPPERKYSV WIGGSILASL
351	STFQQMWISK QEYDESGPSI VHRKCF

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