Biogeosciences Discuss., 10, 2675–2703, 2013
www.biogeosciences-discuss.net/10/2675/2013/
doi:10.5194/bgd-10-2675-2013

Biogeosciences Discussions Open Access

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

E. Martins[1,2], A. Queiroz[3], R. Serrão Santos[1,2], and R. Bettencourt[2]

[1]Department of Oceanography and Fisheries, University of the Azores (DOP/UAç), Rua Prof. Doutor Frederico Machado, 9901-862 Horta, Portugal
[2]Instituto do Mar/Institute of Marine Research (IMAR), Laboratory of Robotics and Systems in Engineering and Science (LARSyS), 9901-862 Horta (Azores) Portugal
[3]Instituto Politécnico de Viana do Castelo (IPVC), Escola Superior Agrária de Ponte de Lima (ESAPL), Refóios do Lima, 4990-706 Ponte de Lima, Portugal

Received: 30 November 2012 – Accepted: 10 December 2012 – Published: 14 February 2013

Correspondence to: R. Bettencourt (raul@uac.pt)

Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

The deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* lives in a natural environment characterized 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* strains. *Flavobacterium* suspensions were also used as an irrelevant *bacterium.* Gene expression analyses were carried out using gill samples from animals dissected at 12 h and 24 h post-infection times 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 and 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 microorganism species 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 around a protein area, of 18 KDa molecular mass, where most dissimilarities were found. Multivariate analyses demonstrated that immune genes, as well as experimental infections, clustered in discrete groups in accordance with the patterns observed in gene expression changes induced by bacterial pathogens.

BGD
10, 2675–2703, 2013

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 allowing the existence of animals and microbes under extreme environments (Duperron et al., 2009).

Deep-sea vent mussels of the *Bathymodiolus* genus are dominant biomass 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) supporting thus 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 characterized 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 first line of host defense against microbial pathogens (Janeway and Medzhitov, 2002; Kumar et al., 2009). This system recognizes conserved molecules of microbial origin found in bacteria, viruses, protozoa and fungi, and known as Pathogen-Associated Molecular Patterns (PAMPs) (Akira and Hemmi, 2003; Medzhitov, 2007), 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 Mollusks 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

BGD
10, 2675–2703, 2013

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 mollusks is phagocytosis (Cheng, 1981; Feng, 1988; May et al., 2001).

Vibrio spp. is the major cause for disease occurring in the marine environment (Demírcan and Candan, 2006). The leading problem is high mortality caused by bacterial pathologies (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). Although *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).

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 different *Vibrio* strains and *Flavobacterium*, were performed and subsequently vent mussel immune gene expression analyzed by qPCR. Additionally, protein profiles were also analyzed 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.

BGD
10, 2675–2703, 2013

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 (POV). 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 B. Allam (Stony Brook University, NY, USA and from A. 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 in a Marine Broth (CaldoDifco™) ($OD_{600} = 1.5$) and added as suspensions, to the 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.

BGD
10, 2675–2703, 2013

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 proteins (PGRP), Serpin, Aggrecan, Lipopolysaccharide (LPS)-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) (LBP-BPI), Immune lectin receptor 2, Toll-like receptor 2, Tumor necrosis factor (TNF) receptor associated 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 along 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.

BGD
10, 2675–2703, 2013

The standard cycle condition used in this study was 95 °C for 10 min, 94 °C for 20 s, 52 °C for 20 s and 68 °C for 30 s followed by 40 cycles of 65 °C for 5 min and 95 °C for 5 min. The gene expression was normalized using a housekeeping gene, the 28S ribosomal gene. Data analysis is based on the Delta-Delta Ct ($\Delta\Delta C_t$) method with normalization of the raw data to housekeeping assays. The Forward and reverse sequences using qPCR are shown in Table 1. Two technical replicates were obtained from qPCR and data was expressed as means and Standard Deviation.

The immune genes analyzed in the present study, were classified according to four categories of functional genes such as Recognition, Signaling and Transcription and Effector (Bettencourt et al., 2010). The primer pairs efficiency (Table 2) were analyzed 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 are expressed as mean ± Standard Deviation (SD). The differences in gene response, 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

BGD
10, 2675–2703, 2013

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

B. azoricus gills infected with marine bacterial pathogens, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, POV, and *Flavobacterium* were used for protein assessment and separation in polyacrylamide gel electrophoresis experiments. Control samples from seawater incubations were also considered. SDS-PAGE analyses were carried out with homogenates consisting of four gills mixtures corresponding to 12 h and 24 h time point infections. 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 100x, 200 µL TritonX-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), with gentle agitation, for 1 h, at 45 °C until protein bands were adequately visualized.

BGD
10, 2675–2703, 2013

3 Results and discussion

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 POV resulted in a slightly above seawater 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). Below seawater gene expression levels were found for the Rhamnose binding lectin, PGRP, Aggrecan, LPB-BPI genes suggesting that *B. azoricus* gills retain a discriminatory capacity while inducing differential transcriptional activities, regarding the microorganism tested in comparison to seawater expression levels. Interestingly, 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,* as opposed to what has been observed for *Flavobacterium* at 12 h. The immune lectin receptor 2 up-regulation is still noticeable for *V. anguillarum* and POV, 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* comparatively to the control (Seawater). On the contrary, down-regulation was observed for the same gene in the presence of *V. splendidus,* POV and

BGD
10, 2675–2703, 2013

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 once more the highest level of expression among the signaling genes tested, in the presence of *V. splendidus* at 24 h in contrast to what has been quantified for *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 POV 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* and also POV (Fig. 1e). On the other hand, infections with *Vibrio* spp. (*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 gene expression levels were found in the presence of *V. alginolyticus*, *V. anguillarum*, POV 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, in the presence of the same bacterium, at 12 h (Fig. 1g). Moreover, effector genes showed down-regulation in the presence of *V. splendidus*, POV, *V. alginolyticus* and *V. anguillarum* (Fig. 1g). The 24 h post-infection transcriptional activity for cytolisin revealed a higher expression level compared to 12 h (Fig. 1h).

Our descriptive data analysis was 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 to provide evidence to whether or not their innate immune system is capable of discriminating different *Vibrio* strains. The hierarchical clustering dendrogram demonstrated how data originated from bacterial infections and from control-seawater conditions were grouped based

BGD
10, 2675–2703, 2013

on the quantitative gene expression pattern they induce. Four clusters were considered through the analysis of bacterial infection conditions cluster dendrogram regarding 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 samples, regarded as our experimental control. The next cluster is formed by POV, *V. anguillarum* and *V. splendidus* and the last cluster is formed by *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 at 24 h incubation time (Fig. 3), three clusters were defined, which in this case included a cluster consisting of the control, a second consisting of *V. splendidus*, and third cluster based upon the remaining *Vibrio*, POV and *Flavobacterium* results This correlates with *V. splendidus* infection, that results often in up-regulated immune genes (Fig. 1b, d, f and h). The hierarchical cluster dendrogram gave a broad view on how marine bacterial pathogens challenges host immune defense.

The multivariate statistical analyses also hinted at how signaling pathways may be activated in *B. azoricus* during immune responses triggered by marine bacteria. Clustering data using hierarchical clustering R analysis, relative to 12 h (Fig. 4) and 24 h post-infection (Fig. 5) gene expression levels, gave rise to a genes cluster dendrogram where fours clusters were evidenced. In Fig. 4 (12 h) one cluster was constituted only by the Toll-like receptor 2 (TLR2) gene. The TLR family includes receptors found both at the cell intracellular surface and they have a role in innate recognition of microbial products (Blasius and Beutler, 2010). The fact that TLR2 is included as 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 by MyD88, Lysozyme, Immune lectin receptor 2 and Jun-like genes. A third cluster includes TRAF6 and Serpin gene, both genes, may be involved in the Toll signaling pathway. TRAF6 mediates signaling and Serpin 2 is a

BGD
10, 2675–2703, 2013

serine-type endopeptidase with inhibitor activity. A fourth cluster was formed by Glutathione peroxidase, Metallothionein, PGRP, LBP-BPI, STAT-SH2, Rhamnose binding lectin, Aggrecan, EGF and Cytolysin genes (Fig. 4).

According to the genes cluster dendrogram regarding results obtained at 24 h incubation time (Fig. 5), four clusters were considered. The first cluster was formed by Immune lectin receptor 2 and second cluster by TLR2. The third cluster was formed by Cytolysin, Glutathione peroxidase and Jun-like. The remaining genes were grouped in a fourth cluster (Fig. 5). Our results suggest that TLR2 is involved in the recognition of different bacterial infectious strains while expressing distinctively according to Vibrio strain and time of infection analyzed. For instance, TLR2 was down-regulated upon *V. anguillarum* incubation and up-regulated 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.

Our statistical analyses also include Heatmaps to illustrate the natural clustering between the experimental conditions, control (Seawater), *V. splendidus, V. alginolyticus, V. anguillarum,* POV and *Flavobacterium* incubations data and the expression of sixteen immune genes at 12 h (Fig. 6), at 24 h post-infection times (Fig. 7).

As in the cluster dendrograms, the TLR2 gene was expressed to its highest level upon *V. alginolyticus* infection which is visualized in the Heatmap as a dark pink color whereas for other *Vibrio* infections, lower levels of expression are represented by other colors. The lowest level of expression is represented by a dark blue color such as in the case of POV infection. The Heatmap corresponding to 24 h post-infection data (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 for control, *V. splendidus* and POV incubations.

Understanding the genes involved in signaling pathways, can give invaluable information, on how animals respond to environmental microorganisms that in the case of vent mussels may involve contact with pathogens from the surrounding vent

BGD
10, 2675–2703, 2013

environment. Nevertheless, our understanding of *B. azoricus* innate signaling pathways is still under investigation by our group.

Our statistical analyses, particularly the hierarchical clustering approaches as dendrograms and/or Heatmaps may prove resourceful for the planning of future experiments involving immune gene expressions that may now be selected on the basis of their relativeness as evidenced in our analyzes by the establishment of distinct cluster groups and how distinct genes were grouped between themselves. There are 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 mediator functions and is associated with innate immunity (Ihle, 2001), while the 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 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.

The SDS-PAGE protein analyses revealed some differences between *B. azoricus* gill samples from the 12 h and the 24 h infection experiments (Fig. 8). 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 was subsequently analyzed by mass spectrometry and identified as actin protein by searches in the NCBI database (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 (Fig. 8).

BGD
10, 2675–2703, 2013

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. 8).

4 Conclusions

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 immune responses researched in our study thus far indicates that vent mussel display varied 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 is , over the course of time and according to the bacteria strain tested. Infections carried out with *Flavobacterium*, induced the highest gene expression levels at 12 h time point, whereas *V. splendidus* infections revealed greater gene expression levels at 24 h time point. This supports to the possibility that *B. azoricus,* when in contact, in their gill tissues, with several bacterial pathogens, may display immune responses, in a selective manner, to counter a variety of bacterial infections.

The genes Dendrogram and Heatmap Plots suggested that genes clusters were connected according to its function in immune signaling pathways. The gene expression profiles changed according to, the bacterial pathogen tested, the primers that were used to target a specific immune gene and the time period of experimental infections. These conditions were statistically confirmed ($p < 0.05$) with Mann-Whitney test and Tukey HSD test. We conclude from this study that *B. azoricus* mussels were under stress conditions, since the control and infections were significantly different. Also the SDS-PAGE analysis, pointed at differences between 12 h and 24 h infection times which were interpreted as a result of an elevated transcriptional status in animals exposed to *Vibrio* spp. for 24 h that in turn ensued in the synthesis of more proteins.

BGD
10, 2675–2703, 2013

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 the 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.

Appendix A

Protein sequence

Matched peptides are shown in **Bold**.

1 MCDEDVAALV VDNGSGMCKA GFAGDDAPRA VFPSIVGRPR HQGVMVGMGQ
51 KDSYVGDEAQ SKRGILTLKY PIEHGIVTNW DDMEKIWHHT FYNELRVAPE
101 EHPVLLTEAP LNPKANREKM TQIMFETFNS PAMYVAIQAV LSLYASGRTT
151 GIVLDSGDGV SHTVPIYEGY ALPHAIIR**LD LAGRDLTDYQ MKILTERGYS**
201 **FTTTAEREIV R**DIKEKLCYV ALDFEQEMQT AASSSSLEK**S YELPDGQVIT**
251 **IGNER**FRAPE ALFQPSFLGM ESAGVHETTF NSIGKCDIDI RK**DLYANTVL**
301 **SGGTTMFPGI ADRMQKEISA LAPPTMK**IKI IAPPERKYSV WIGGSILASL
351 STFQQMWISK **QEYDESGPSI VHRK**CF

Acknowledgements. We are grateful to the Portuguese Foundation for Science and Technology (FCT) for Doctoral grant (SFRH/BD/68951/2010) and Bassem Allam (Stony Brook University, NY, USA), Antonio Figueras, (CSIC, IIM, Vigo, Spain) that kindly provided the Vibrios Strains used in this study and our colleague Sergio Stefanni for helpfull comments. We acknowledge the HERMIONE project grant agreement no. 226354. IMAR-DOP/UAz (research and Development Unit #531) and LARSyS Associated Laboratory are supported by FCT (under the strategic project (OE, COMPETE, QREN) and by DRCT – Regional Government of the Azores through a Pluriannual Funding scheme. This work was also supported by the FCT research project IMUNOVENT PTDC/MAR/65991/2006 (granted to RB). The service charges for this open ac-

BGD
10, 2675–2703, 2013

cess publication have been covered by “LARSyS Associated Laboratory through FCT/MCE project PEst-OE/EEI/LA0009/2011”.

References

Akira, S. and Hemmi, H.: Recognition of pathogen-associated molecular patterns by TLR family, Immunol. Lett, 85, 85–95, 2003.

Beaz-Hidalgo, R., Balboa, S., Romalde, J. L., and Figueras, M. J.: Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs, Environ Microbiol. Report., 2, 34–43, 2010.

Bettencourt, R., Roch, P., Stefanni, S., Rosa, D., Colaço, A., and Santos, R. S.: Deep sea immunity: unveiling immune constituents from the hydrothermal vent mussel *Bathymodiolus azoricus*, Mar. Environ. Res., 64, 108–127, 2007.

Bettencourt, R., Dando, P., Collins, P., Costa, V., Allam, B., and Serrão Santos, R.: Innate immunity in the deep sea hydrothermal vent mussel *Bathymodiolus azoricus,* Comp. Biochem. and Physiol., 152, 278–289, 2008.

Bettencourt, R., Pinheiro, M., Egas, C., Gomes, P., Afonso, M., Shank, T., and Santos, R.: High-Throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*, BMC Genomics, 11, 559–576, 2010.

Blasius, A. L. and Beutler, B.: Intracellular Toll-like Receptors Rev., Immunity Cell Press, 32, 305–315, 2010.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T.: The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-time Experiments Rev., Clin. Chem., 55, 554–611, 2009.

Cheng T. C.: Bivalves in invertebrate blood cells, edited by: Ratcliffe, N. A. and Rowley, A. F., London Academic Press, 233–299, 1981.

Childress, J. J. and Fisher C. R.: The biology of hydrothermal vent animals: physiology, biochemistry, and autotrophic symbioses, Oceanogr Mar Biol. Annu Rev., 30, 337–441, 1992.

Colaço, A., Bettencourt, R., Costa, V., Lino, S., Lopes, H., Martins, I., Pires, L., Prieto, C., and Serrão Santos, R.: LabHorta: a controlled aquarium system for monitoring physiological characteristics of the hydrothermal vent mussel *Bathymodiolus azoricus*, Ices J. Mar. Sci., 68, 349–356, 2010.

BGD
10, 2675–2703, 2013

De Chaine, E. G., Bates, A. E., Shank, T. M., and Cavanaugh, C. M.: Off-axis symbiosis found: Characterization and biogeography of bacterial symbionts of *Bathymodiolus mussels* from Lost City hydrothermal vents, Environ. Microbiol., 8, 1902–1912, 2006.

Demírcan, D. and Candan, A.: Identification of Vibrio anguillarum by PCR (rpoN Gene) Associated with Vibriosis in Marine Fish in Turkey, Turk. J. Vet. Anim. Sci., 30, 305–310, 2006.

Duperron, S. Lorion, J., Samadi, S., Gros, O., and Gaill, F.: Symbioses between deep-sea mussels (Mytilidae: Batymodiolinae) and chemosynthetic bacteria: diversity, function and evolution, C.R. Biologies, 332, 299–310, 2009.

Esko, J. D., Kimata, K., and Lindahl, U.: Proteoglycans and Sulfated Glycosaminoglycans Chap. 16 Essentials of Glycobiology, 2nd ed., edited by: Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hard, G. W., and Etzler, M. E., Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2009.

Feng, S. Y.: Cellular defense mechanisms of oysters and mussels, Am. Fish. S., Special Publication, 18, 153–168, 1988.

Galloway, T. and Depledge, M.: Immunotoxicity in invertebrates: measurement and ecotoxicological relevance, Ecotoxicological, 10, 5–23, 2001.

Gómez-León, J., Villamil, M. L., Novoa, B., and Figueras, A.: Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from Aquacultured Carpet Shell Clam (*Ruditapes decussatus*), Larvae Associated with Mass Mortalities, Appl. Environ. Microbiol., 71, 98–104, 2005.

Grum, M. and Atieno, F.: Statistical analysis for plant genetic resources: clustering and indices in R made simple, Handbooks for Genebanks, No. 9, Bioversity International, Rome, Italy, 2007.

Ihle, J. N.: The Stat family in cytokine signaling, Cur. Opin. Cell Biol., 13, 211–217, 2001.

Janeway, J. and Medzhitov R.: Innate immune recognition, Ann. Rev. Immunol., 20, 197–216, 2002.

Kumar, H., Kawai, T., and Akira, S.: Pathogen recognition in the innate immune response, Biochem J. Rev. Art., 420, 1–16, 2009.

Labreuche, Y. Lambert, C., Soudant, P. Boulo, V., Huvet, A., and Nicolasa, J.-L.: Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32, Microb. Infect., 8, 2715–2724, 2006.

Livak, K. and Schmittgen, T. D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods, 25, 402–408, 2001.

BGD
10, 2675–2703, 2013

May, R. C. and Machesky, L. M.: Phagocytosis and the actin cytoskeleton, J. Cell Sci., 114, 1061–1077, 2001.
Medzhitov, R.: Toll-like receptors and Innate immunity, Nature Rev. Immnol., 1, 134–145, 2001.
Medzhitov, R.: Recognition of microorganisms and activation of the immune response, Insight Rev. Nature, 449, 819–826, 2007.
Paillard, C., Le Roux, F., and Borrego, J.: Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution, Aquat. Living Resour., 17, 477–498, 2004.
Salerno, J., Macko, S. A., Hallam, S. J., Bright, M., Won, Y., Mckiness, and Van Dover, C.: Characterization of Symbiont Populations in Life-History Stages of Mussels from Chemosynthetic Environments, Bull. Marine Biol. Lab., 208, 145–155, 2005.
Tanabe, K. K., Pharm, A. L., Finkelstein, D. M., Kawasaki, H., Fujii, T., Chung, R. T., Lauwers, G. Y., Kulu, Y., Muzikansky, A., Kuruppu, D., Lanuti, M., Goodwin, J. M. Azoulay, D., and Fuchs, B. C.: Epidermal Growth Factor Gene Functional Polymorphism and the Risk of Hepatocellular Carcinoma in Patients With Cirrhosis, J. AM. Med. Assoc., 299, 1, 53–60, 2008.
Teske, A.: Deep-Sea Hydrothermal vents, The Desk Encyclopedia of Microbiology 2nd Edition by Moselio Schaechter, Academia Press, 346–356, 2009.
Toranzo, A. E. and Barja, J. L.: A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the northwest of Spain, Diseas. Aquat. Org., 9, 73–82, 1990.
Velleman, S. G.: The Role of the Extracellular Matrix in Skeletal Development, Poult. Sci., 79, 985–989, 2000.

BGD
10, 2675–2703, 2013

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

BGD
10, 2675–2703, 2013

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

BGD
10, 2675–2703, 2013

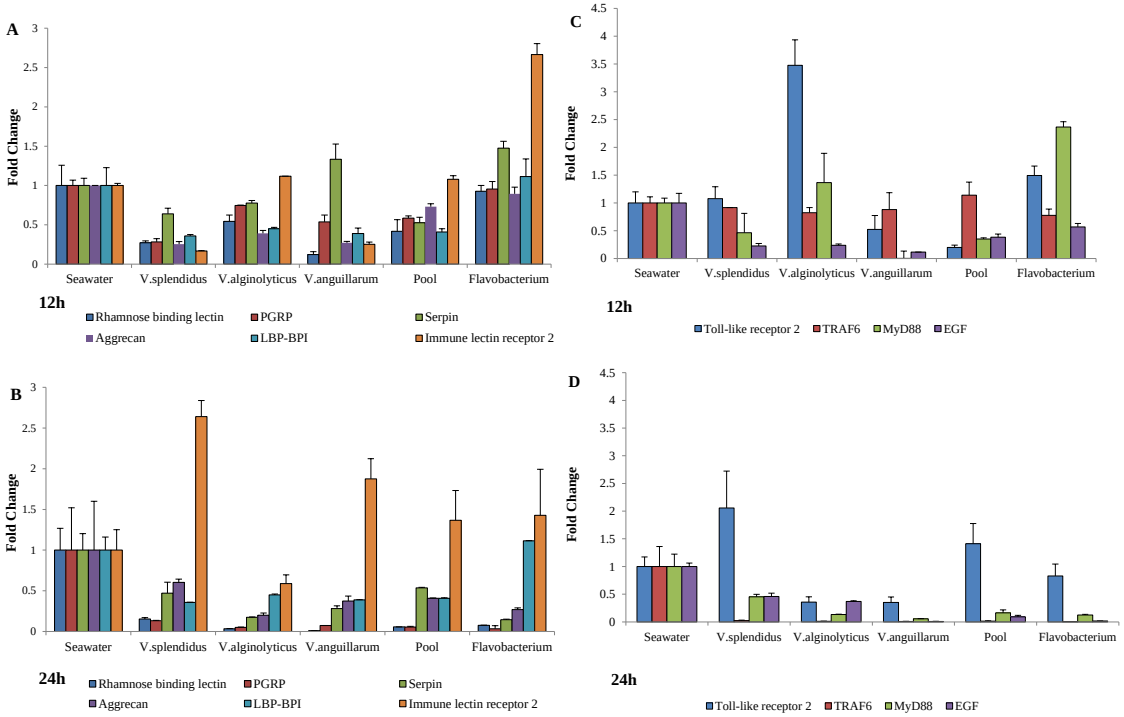

Fig. 1. Differential expression of immune genes. **(A)** Expression levels of immune recognition genes (Rhamnose-binding lectin, PGRP, Serpin, Aggrecan, LBP-BPI and Immune lectin receptor 2) from *B. azoricus* infected gills at 12 h infection time. **(B)** Expression levels of immune recognition genes 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 normalized to the housekeeping gene 28S.

BGD
10, 2675–2703, 2013

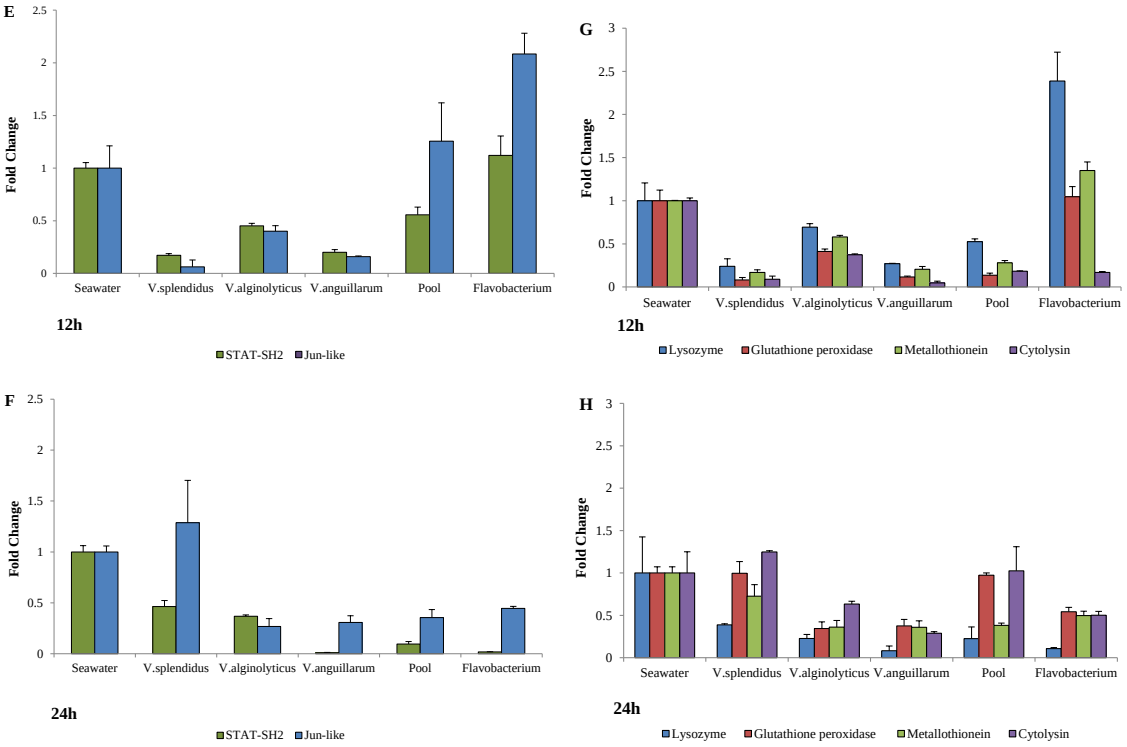

Fig. 1. Continued.

BGD
10, 2675–2703, 2013

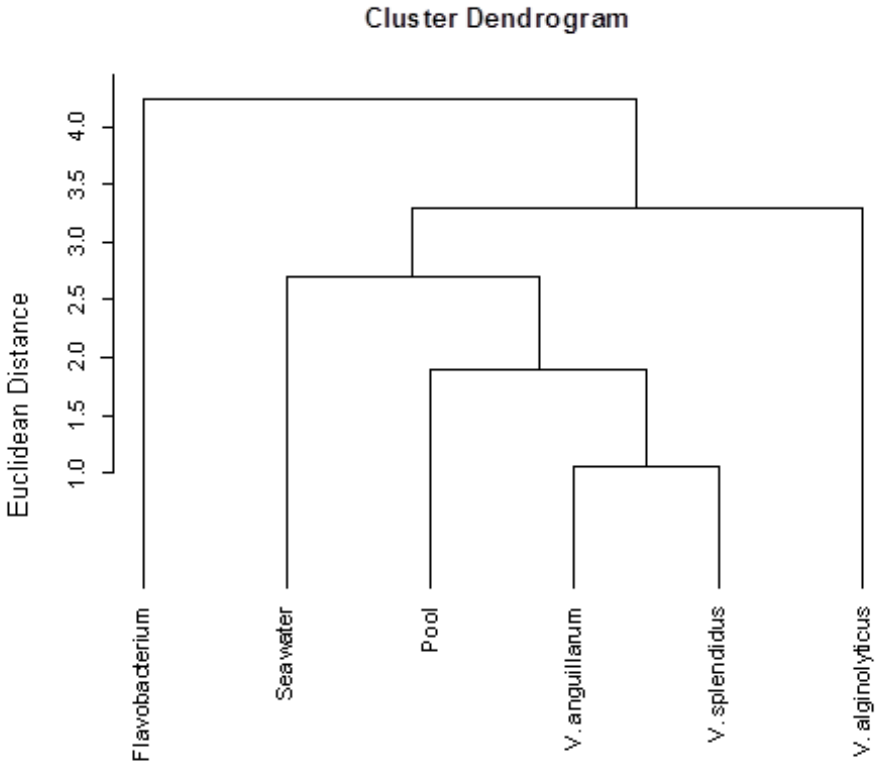

Fig. 2. Hierarchical clustering Dendrogram of Experimental condition (*Flavobacterium*, POV, *V. anguillarum*, *V. splendidus*, *V. alginolyticus* and Control (Seawater) of mussel *B. azoricus* at 12 h post-infection through Euclidean distance, using UPGMA method.

BGD
10, 2675–2703, 2013

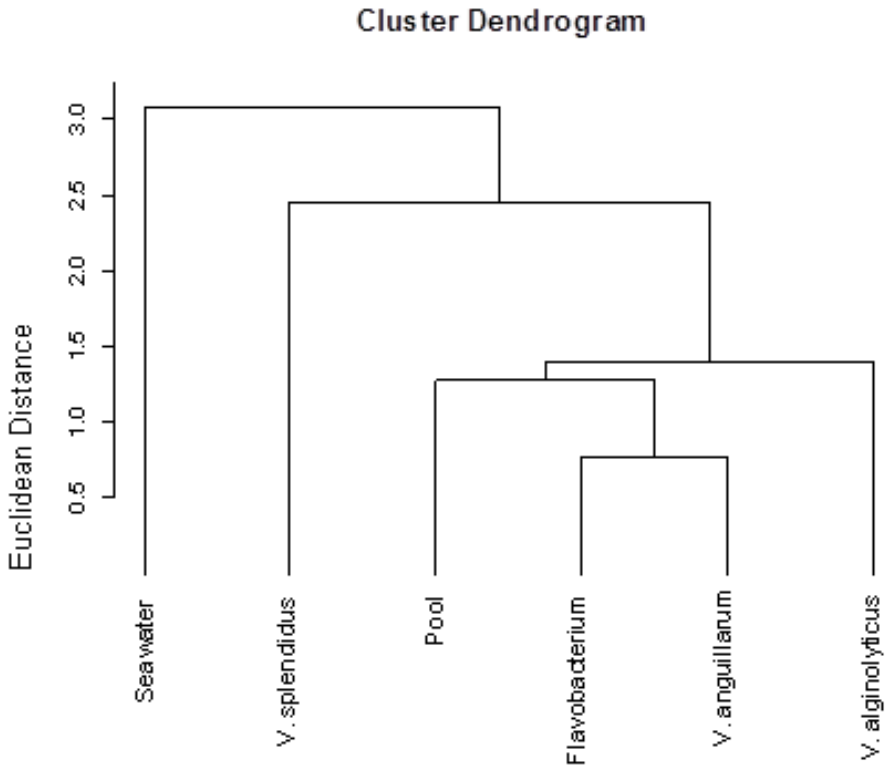

Fig. 3. Hierarchical clustering Dendrogram of Experimental condition (*Flavobacterium*, POV, *V. anguillarum*, *V. splendidus*, *V. alginolyticus* and Control (Seawater) of mussel *B. azoricus* at 24 h post-infection through Euclidean distance, using UPGMA method.

BGD
10, 2675–2703, 2013

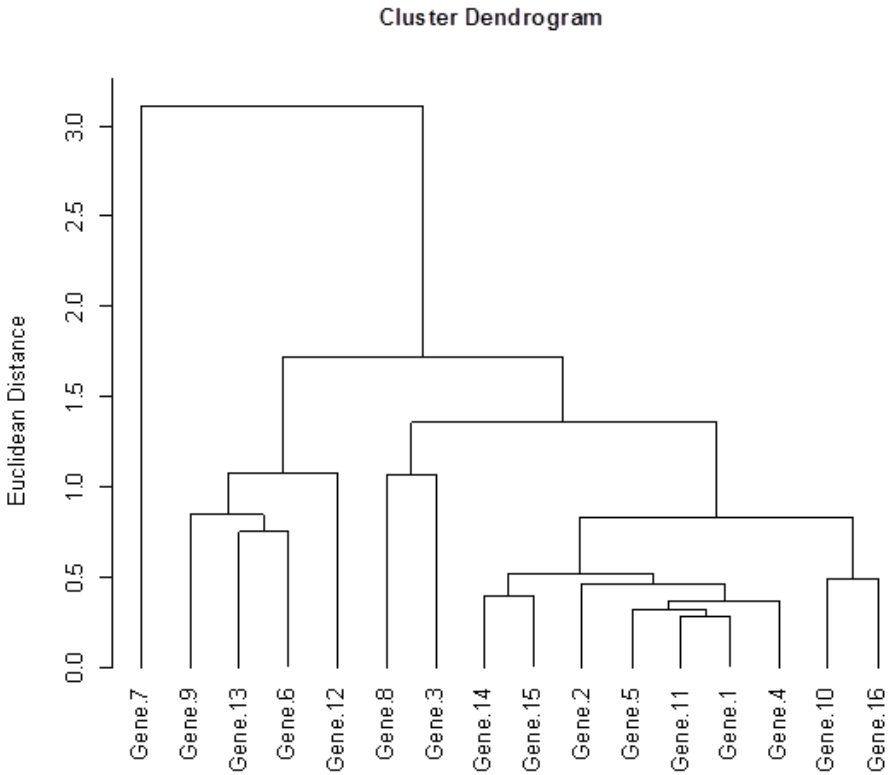

Fig. 4. Gene Cluster Dendrogram of mussel *B. azoricus* at 12 h post-infection through Euclidean distance, using UPGMA method. The numeration of Genes represent: (1) Rhamnose binding lectin; (2) PGRP; (3) Serpin; (4) Aggrecan; (5) LBP-BPI; (6) Immune lectin receptor 2; (7) Toll-like receptor 2; (8) TRAF6;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

BGD
10, 2675–2703, 2013

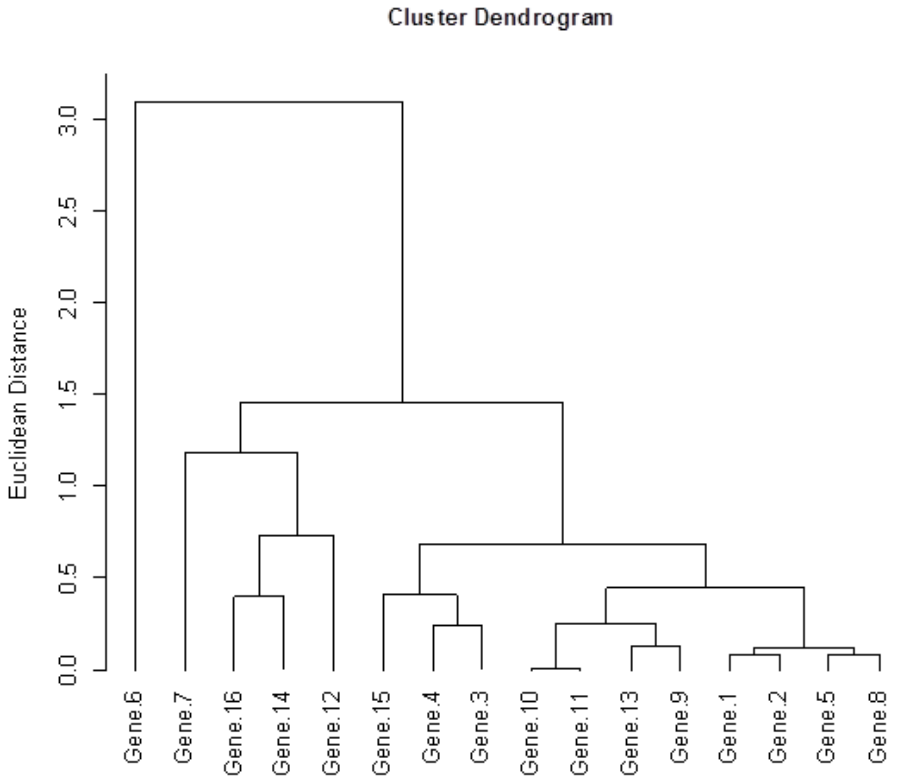

Fig. 5. Gene Cluster Dendrogram of mussel *B. azoricus* at 24 h post-infection through Euclidean distance, using UPGMA method. The numeration of Genes represent: (1) Rhamnose binding lectin; (2) PGRP; (3) Serpin; (4) Aggrecan; (5) LBP-BPI; (6) Immune lectin receptor 2; (7) Toll-like receptor 2; (8) TRAF6;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

BGD
10, 2675–2703, 2013

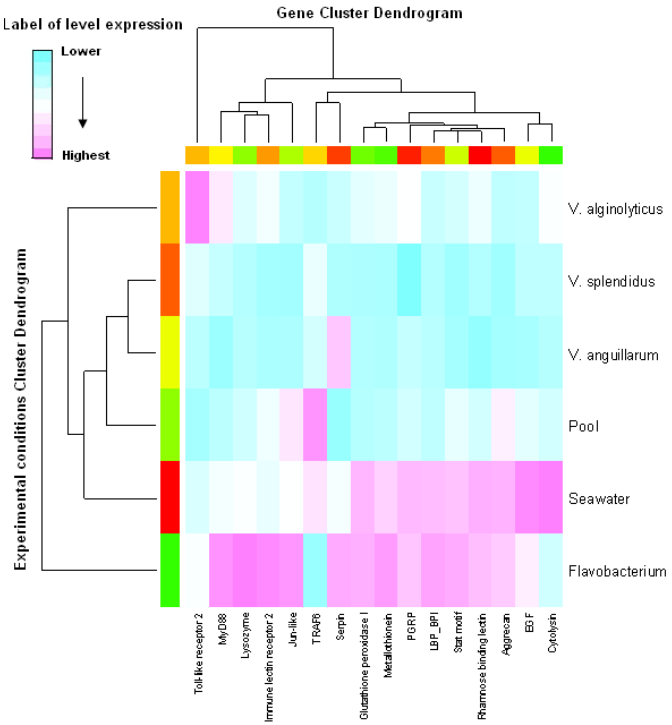

Fig. 6. Hierarchical Clustering Heatmap Plot of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The heatmap represents a grid of colored points where each color represents a level of gene expression (Fold change). The rows represent Experimental conditions (*V. alginolyticus*,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

BGD
10, 2675–2703, 2013

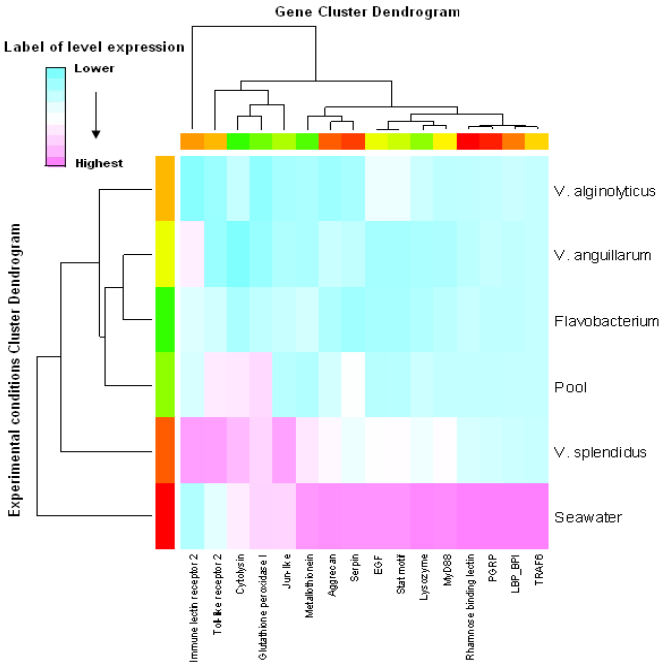

Fig. 7. Hierarchical Clustering Heatmap Plot of dendrograms using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The heatmap represents a grid of colored points where each color represents a level of gene expression (Fold change). The rows represent Experimental conditions (*V. alginolyticus*,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

BGD
10, 2675–2703, 2013

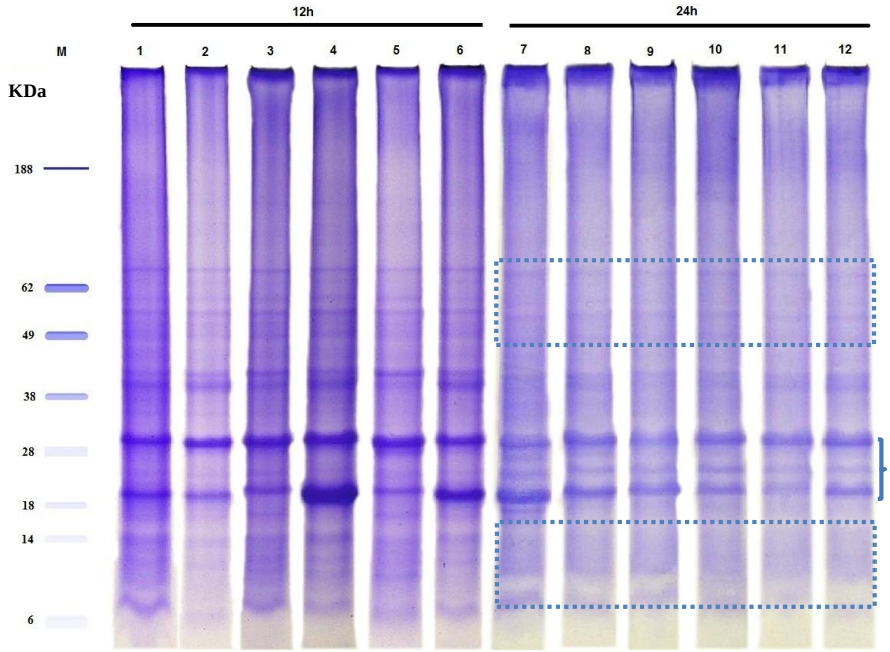

Fig. 8. SDS-PAGE of mix gills protein samples from *B. azoricus,* incubated in the presence of Seawater, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, pool of Vibrio and *Flavobacterium*. The lanes 1, 2, 3, 4, 5 and 6 represent samples at 12 h infection and the lanes 7, 8, 9, 10, 11 and 12 correspond 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.

BGD
10, 2675–2703, 2013