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The immune response of the scallop *Argopecten purpuratus* is associated with changes in the host microbiota structure and diversity

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ABSTRACT

All organisms live in close association with a variety of microorganisms called microbiota. Furthermore, several studies support a fundamental role of the microbiota on the host health and homeostasis. In this context, the aim of this work was to determine the structure and diversity of the microbiota associated with the scallop *Argopecten purpuratus*, and to assess changes in community composition and diversity during the host immune response. To do this, adult scallops were immune challenged and sampled after 24 and 48 h. Activation of the immune response was established by transcript overexpression of several scallop immune response genes in hemocytes and gills, and confirmed by protein detection of the antimicrobial peptide big defensin in gills of *Vibrio*-injected scallops at 24 h post-challenge. Then, the major bacterial community profile present in individual scallops was assessed by denaturing gradient gel electrophoresis (DGGE) of 16S rDNA genes and dendrogram analyses, which indicated a clear clade differentiation of the bacterial communities noticeable at 48 h post-challenge. Finally, the microbiota structure and diversity from pools of scallops were characterized using 16S deep amplicon sequencing. The results revealed an overall modulation of the microbiota abundance and diversity according to scallop immune status, allowing for prediction of some changes in the functional potential of the microbial community. Overall, the present study showed that changes in the structure and diversity of bacterial communities associated with the scallop *A. purpuratus* are detected after the activation of the host immune response. Now, the relevance of microbial balance disruption in the immune capacity of the scallop remains to be elucidated.

1. Introduction

Chile is among the top ten aquaculture producers in the world [1]; mollusks are the third most important aquatic resource in the country, and they have a great impact on the local economy. Scallop rearing is associated with high population density which can affect the immune function of these animals, making them susceptible to infectious diseases [2]. Indeed, production of the scallop *Argopecten purpuratus* has declined, in part due to massive mortalities in larvae caused by the gram-negative pathogen *Vibrio splendidus* [3]. Although in *A. purpuratus* these mortalities are still mainly recognized as a larval problem, pathogenic vibrios have been registered for adult scallops of other species. For instance, *V. splendidus* has been identified as a pathogenic agent with fatal consequences in adult scallops of *Patinopecten yessoensis* [4]. For this reason, understanding the underlying mechanisms related to the immune capacity of reared aquatic organisms has become an important challenge for sustainable aquaculture production [5].

Traditionally, the immune response of all invertebrates has been considered to rely mainly on mechanisms of innate immunity, mediated by cellular and humoral components [6]. In bivalve mollusks, immunity provides protection against pathogenic organisms and environmental stressors, such as the presence of contaminants, changes in temperature or salinity or mechanical stress [7]. The immune response of bivalves is carried out by the immunocompetent hemocytes, and by mucosal surfaces such as gills and mantle tissues. These tissues participate in the recognition of non-specific molecules by soluble and membrane bound pattern recognition proteins (PRPs) and receptors (PRRs), respectively, which activate intracellular signaling pathways. Subsequently, this recognition triggers cellular and humoral immune responses such as hemocyte phagocytosis and expression of key antimicrobial effectors [8].

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In particular, several immune genes coding for immune signaling pathways [9] and molecular effectors [10–12] have been recently characterized in the scallop *A. purpuratus*, increasing the knowledge on the immune response in this species.

Currently, several studies have recognized that the associated microbiota of every metazoan might play an important role in the immune response capacity [13,14]. In turn, the host-associated bacterial communities can participate in various physiological processes, including protection from environmental changes [15]; conduct metabolic processes that the host cannot perform [16]; influence the development of organs through chemical signals [17], and participate in the development of the immune system [18]. Hence, the current evidence indicates that the interaction between the host and associated microbiota is essential for maintenance of host homeostasis.

The emerging view of the immune defense mechanisms in bivalve mollusks suggests a strong interplay between the host immune system and its associated microbiota present in the host [5,14]. Indeed, the importance of bacterial communities associated with the host, and the effects of their imbalance on host health, has been described in some marine bivalves such as oysters [15,19]. Recently, the microbiota from clams [20], abalones, mussels, sea urchins [21] and the scallop *Patinopecten yessoensis* [22] has been characterized using 16S rDNA deep amplicon sequencing. Although some cultivable-dependent bacterial studies are found in *A. purpuratus* (revised by Ref. [23]), no data on the total microbiota is available in this species. Bivalve mucosal surfaces and the marine environment are both complex ecosystems that display different and diverse bacterial communities, both commensal and potentially pathogenic [19,24]. Yet, healthy scallops are colonized by abundant microbial communities that coexist with the hemocytes in the circulatory system. If scallops are capable of subsisting in such environment, specific immune mechanisms could be participating in a coordinated manner to maintain scallop homeostasis. For instance, certain immune effectors may be involved because of their recognized role as antimicrobial molecules. In this context, as a first step in understanding the interplay between the scallop immune effectors and the microbiota, it is necessary to evaluate if the bacterial composition changes after the immune response activation.

In this study, the bacterial communities associated with the scallop *A. purpuratus* were examined by denaturing gradient gel electrophoresis (DGGE) and 16S rDNA deep amplicon sequencing before and after the host immune stimulation. The results obtained in this study constitute, to the best of our knowledge, the first evidence of an association between changes on the composition and diversity of bacterial communities of a scallop following immune response.

2. Materials and methods

2.1. Animals, bacterial challenge and tissue collection

US National Research Council guidelines for the care and use of laboratory animals were strictly followed during this research [25]. Adults scallops (60–70 mm shell height) were sampled from the aquaculture facilities of the Universidad Católica del Norte (UCN) located in Tongoy Bay, Chile (30°16′ S, 71° 35′W). One hundred microliters of heat-killed *Vibrio splendidus* VPAP16 [3] (VS) in 0.22 μm filtered sea water (FSW) ($1x10^6$ cells/scallop) or 100 μL of FSW (as injury control) were injected in the adductor muscle of 20 scallops per condition. This dose of heat-killed *V. splendidus* has been previously validated as an immune response activator in *A. purpuratus* [11]. In addition, 10 naive scallops were included as a control (NI, non-injected). Each experimental condition and time point included 10 animals in the analysis.

The experiment was rigorously designed to minimize the effects of spatial and temporal variability. All scallops were placed in the same lantern net on July 1, 2015, each group separately (water temperature: 13 °C, depth: 5–10 m). At this time point, the first group was injected (FSW48 and VS48). After 24 h, the lantern net was removed from the sea and the second group was injected (FSW24 and VS24). After 24 h, the lantern net was removed from the sea and the three groups (NI, 24 and 48) were collected and transferred to the UCN laboratory in Coquimbo, Chile. A hemolymph subsample of 1 ml was individually collected from the pericardial cavity, the hemocytes were isolated by centrifugation (600 × g for 5 min at 4 °C) and kept in TRIzol® reagent at −80 °C until total RNA extraction. Two subsamples of gills (5 mm^2 each) from the same scallops were harvested by dissection under sterile conditions and kept in TRIzol® reagent at −80 °C until total RNA extraction or Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) for immunofluorescence. After the subsampling, whole scallops were removed from the shell, individually snap-frozen in liquid nitrogen and maintained at −80 °C until total genomic DNA extraction.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted from *A. purpuratus* circulating hemocytes and gill tissues using TRIzol® reagent according to the manufacturer's instructions (Thermo Scientific). RNA was then treated with DNase I (Thermo Scientific), 15 min at room temperature and inactivated by heat, 10 min at 65 °C, followed by a second precipitation with sodium acetate 0.3 M (pH 5.2) and isopropanol (1:1 v:v). The pellet was reconstituted in ultra-pure water and stored at −80 °C. The quantity and quality of total RNA were assessed using a NanoDrop® spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis, respectively. Synthesis of cDNA was carried out individually using 1 μg of total RNA with the Affinity Script qPCR cDNA Synthesis Kit according to the manufacturer's instructions (Stratagene).

2.3. Real-time quantitative PCR (qPCR) analysis of gene expression

The gene expression analysis was determined through relative quantification by RT-qPCR. The relative expression levels of 11 immune genes were assessed for each cDNA in triplicate on a Stratagene MX30009P (Agilent Technologies®) using 1 × Brilliant III Ultra-Fast SYBR® QRT-PCR Master Mix (Stratagene), 0.3 μM of each primer and 1 μL of cDNA, diluted 1:5. Primers are listed in Table S1. Primer pair efficiencies were calculated from the given slopes in the Stratagene MX30009P software according to the equation: $E = 10^{[-1/slope]}$, and only primer pairs with efficiency between 92 and 106% were used. The assays were submitted to an initial denaturation step of 5 min at 95 °C followed by amplification of the target cDNA (40 cycles of denaturation at 95 °C for 10s, annealing at 57 °C for 10s and extension time at 60 °C for 10s) and fluorescence detection. After an initial 10s denaturation step at 95 °C, a melting curve was obtained from a start temperature of 65 °C to a final temperature of 95 °C, with an increase of 0.06 °C/s. Relative expression was calculated with the $-2^{\Delta\Delta Cq}$ method [26], using the measured quantification cycle (Cq) values of the constitutively expressed genes β-actin (GenBank no. ES469330) and EF-1α (GenBank FE896009) to normalize the measured Cq values of target genes. The NI scallops were considered as the control group for the $-2^{\Delta\Delta Cq}$ determination. Calculations of the means, standard deviations and statistical analyses comparing each experimental condition to the control group, one at a time, were performed using the Kruskal-Wallis test included in the GraphPad Prism software version 6.01 ($P < 0.05$).

2.4. Protein detection of big-defensin by immunofluorescence

Fixed gills were dehydrated through an ascending ethanol series, embedded in Histosec (Merck) and mounted on glass slides. Paraffin sections (7 μm) were cleared in Neo-Clear (Merck) and hydrated in a descending ethanol series. Big defensin immunofluorescence detection was carried out as described previously [11]. Briefly, paraffin sections were incubated with 50 mM NH4Cl for quenching the autofluorescence, incubated overnight at 4 °C with anti-ApBD1 (1:100) in 1% BSA, and then incubated for 1 h with Goat anti-Mouse Alexa Fluor 568-conjugate

(Thermo Scientific) (1:200) in 1% BSA. TO-PRO®-3 Iodide (Thermo Scientific) (1:1000) was used for nuclear staining. Control slides were incubated with a mouse pre-bleed serum. Slides were analyzed using a Leica TCS SP5II spectral confocal microscope (Leica Microsystems).

2.5. Genomic DNA extraction

Frozen scallops were powdered in liquid nitrogen using a mortar and pestle. The DNA extractions were performed individually in the first step of mechanical lysis using zirconium beads and a FastPrep® homogenization, followed by DNA extraction using the E.Z.N.A.® Soil DNA Kit according to the manufacturer's instruction. The quantity and quality of genomic DNA were assessed using a NanoDrop® spectrophotometer (Nanodrop Technologies) and agarose gel electrophoresis, respectively.

2.6. Bacterial community analysis through denaturing gradient gel electrophoresis (DGGE)

For DGGE analysis, the V6–V8 region of the 16 rDNA was amplified by PCR from 8 individuals per experimental condition with the F984GC and R1378 primer set [27]. The samples were separated through polyacrylamide gel electrophoresis in TAE buffer with a denaturation gradient of 30%–70% (with urea and deionized formamide), using the same quantity of PCR product for each sample, at 88 V at 58 °C for 15 h using the DCode™ Universal Mutation Detection System (Bio-Rad). The DGGE band profiles were analyzed using CLIQS 1D Pro software (TotalLab). Background noise was subtracted and the bands were automatically detected at 2% tolerance, then, further corrected and equalized to create a binary absence/presence matrix. Similarities were established using the Dice coefficient [28]. Based on the band presence/absence and band weighting (band density) analyses, the phylogenic dendrogram was constructed by applying the Dice coefficient and the unweighted pair-group method with the use of arithmetic averages (UPGMA). Richness (S) values were calculated as the number of DNA bands detected in the respective line of the DGGE profile, while the Shannon index (H) was calculated according to the equation $H' = -\Sigma(pi)\,(lnpi)$, where pi is the ratio between the specific band intensity and the total intensity of all bands in each sample [29]. Analysis of variance was used to compare indices of diversity and richness in Microsoft Excel. Differences were considered statistically significant at $P < 0.05$.

2.7. Deep amplicon sequencing of the 16S rDNA gene

An equimolar pool of gDNA from 10 scallops from each of the 5 experimental conditions (5 pools in total) was constructed and the 16S rDNA gene of bacterial communities was amplified and sequenced using the variable regions V3–V4 (341F: 5′-CCTACGGGNGGCWGCAG-3'; 805R: 5′-159 GACTACHVGGGTATCTAATCC-3′). Paired-end sequencing (2x300 bp read length) was performed by Macrogen Inc. on a MiSeq system (Illumina®) using the MiSeq Reagent Kit v3 according to the manufacturer's instruction. Raw sequence data are available in the SRA database BioProject ID PRJNA517333.

2.8. 16S rDNA deep amplicon sequencing analysis

Raw reads were processed using QIIME2 (version 2018.6, http://qiime2.org) and developed based on standards described by Ref. [30] for microbiota community evaluation. Demultiplexed paired-end reads were imported as artifacts and denoised using DADA2 [31]. In this step of the analysis, quality control as well as phiX reads (commonly present in marker gene Illumina sequence data) and chimera sequence filtering were applied to ensure the retainment of only high-quality reads. Amplicon sequence variants (ASVs), a higher resolution analog to operative taxonomic units (OTUs) [32], were obtained and further processed for taxonomic assignment and diversity analysis. To avoid retaining rare features present due to sequencing technical issues, features with less than 0.1% of the mean sample depth were filtered out as low confidence [33]. Taxonomy was assigned based on the Green Genes database (gg-13_8 99%) [34,35]; features assigned to the class Chloroplast (Phylum Cyanobacteria) were filtered out due to possible plant origin. Samples were rarefied to the maximum depth of the sample with less sequencing depth, and rarefaction curves were plotted. Alpha diversity was evaluated using the Shannon Diversity Index, and beta diversity was evaluated by assessing weighted and unweighted UniFrac distances [36,37]. A principal coordinate analysis was performed to visualize phylogenetic beta diversity. PICRUST was used to predict the functional profile of each group's microbiota [38]. Differences between groups were evaluated for taxonomic abundance and KEGG pathways prediction using Fisher's exact test [39] with Benjamini-Hochberg FDR [40] correction on STAMP [41].

3. Results

3.1. Immune response

3.1.1. Assessment of scallop immune activation by analysis of gene expression by RT-qPCR

The relative expression of 11 immune-related genes was assessed in control and challenged scallops to determine the activation of the scallop immune response (Fig. 1, Table S1). The panel of genes included PRR/PRPs, a member of the NF-kB immune signaling pathway, antioxidant enzymes and immune effectors (Fig. 1). Results showed that 4 of the 11 genes were significantly overexpressed in hemocytes at 24 h after the *Vibrio* injection, all corresponding to immune effectors (Fig. 1). The transcript expression of the antimicrobials big defensin (*ApBD1*) and lysozyme G (*Glys*), the heat shock protein 70 (*Hsp70*) and the antioxidant enzyme peroxiredoxin (*PRX*) increased 7-, 5-, 3- and 3 folds, respectively, compared with non-injected (NI) control scallops ($P < 0.05$). Similarly, the transcript expression of *ApBD1* and *Glys* increased 8- and 10- folds, respectively, compared with the NI group in gills at 24 h after the *Vibrio* injection (Fig. 1). The extracellular copper-zinc superoxide dismutase enzyme (*eSOD*) was overexpressed in gills with a 7- fold change compared with the NI group. No significant changes in expression between FSW injected scallops and NI scallops were detected in both tissues ($P < 0.05$). The only effector which showed a significant overexpression at 48 h after challenge was the AMP *ApBD1* in gills.

3.1.2. Detection of protein expression of ApBD1 in immune stimulated scallops

Since increased gene expression of *ApBD1* was observed in hemocytes and gills from immune stimulated scallops, further investigation was performed to assess ApBD1 at the protein level. To do this, the protein localization of ApBD1 was assessed in gills by immunofluorescence using an anti-ApBD1 polyclonal antibody [11]. ApBD1 abundantly coated the gills of *A. purpuratus* at 24 and 48 h after the immune challenge (Fig. 2). Furthermore, ApBD1 was only detected in *Vibrio*-challenged scallops and not is FSW-injected scallops or control scallops. These results demonstrate that scallops respond to immune stimulation with the production of immune effectors such as the AMP ApBD1.

3.2. Microbiota

3.2.1. DGGE profiles from single scallops and dendrogram cluster analysis

After validation of the scallop immune activated status, the changes in diversity and composition of the predominant bacterial community associated with individual scallops was explored. For this, dendrogram construction and cluster analysis was performed from the DGGE profiles of 8 individuals per experimental condition. The UPGMA cluster

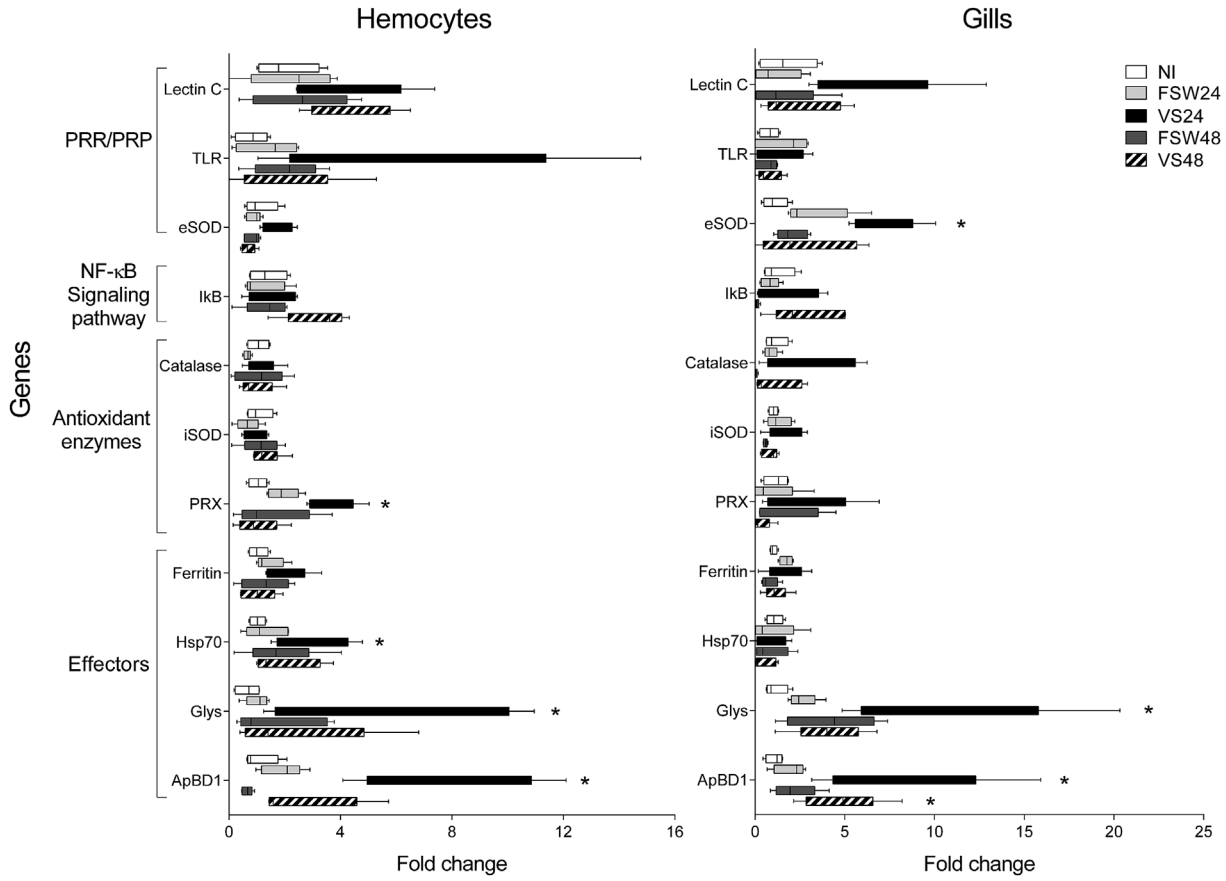


Fig. 1. Relative expression of immune genes in circulating hemocyte and gill tissues from the scallop *Argopecten purpuratus* in response to immune challenge. The scallop β-actin and EF-1α genes were used as reference genes. Relative expressions were obtained from injury stressed scallops (FSW, light gray and dark gray bars) and *Vibrio* challenged scallops (*V. splendidus*, black and lined bars) at 24 and 48 h. Non-injected scallops (NI, white bars) were included. Results are expressed as mean values ± SD, n = 8 by each condition. Asterisks indicate significant differences ($P < 0.05$).

analysis revealed that the composition of the bacterial community from individual NI scallops formed one distinct clade separately from injected scallops, showing more than 80% similarity among NI individuals. Furthermore, no distinct clades between DGGE profiles from FSW24 and VS24 scallops were detected, and accordingly, individuals from both conditions were clustered together (Fig. 3). Notably, the similarity coefficient indicated that every individual from the NI, FSW24 and VS24 groups shared a minimum resemblance of 77%. At 48 h post injection, divergent and distinct clades formed by individuals from the FSW and VS groups were found. The similarity coefficient showed that individuals from the VS48 group shared a resemblance of 75% within each group. This clade displayed less than 50% similarity with the NI, FSW24 and VS24 groups. Individuals from the FSW48 group showed 82% similarity and less than 50% similarity with individuals from the other conditions. Furthermore, the diversity indices of Shannon (H′) and species richness were estimated, showing that both indices significantly decreased in the VS48 group compared with the NI group. Also, a significant decrease of species richness was found in FSW24 and H′ in VS24 groups compared with the NI group (Fig. 3).

3.2.2. Characterization of bacterial communities associated with A. purpuratus by 16S rDNA deep amplicon sequencing analysis

After identifying the changes of the major bacterial groups in immune-activated scallops by DGGE analysis, the overall changes were characterized and which bacterial groups were modulated during the immune response were identified. From deep amplicon sequencing, a total of 2,804,243 raw paired-end reads were quality filtered, denoised and merged, resulting in a total of 254,834 bacterial 16S rDNA gene sequences from *A. purpuratus*, ranging between 37,400 and 55,648 reads per sample (Table S2). Sequences were randomly subsampled and

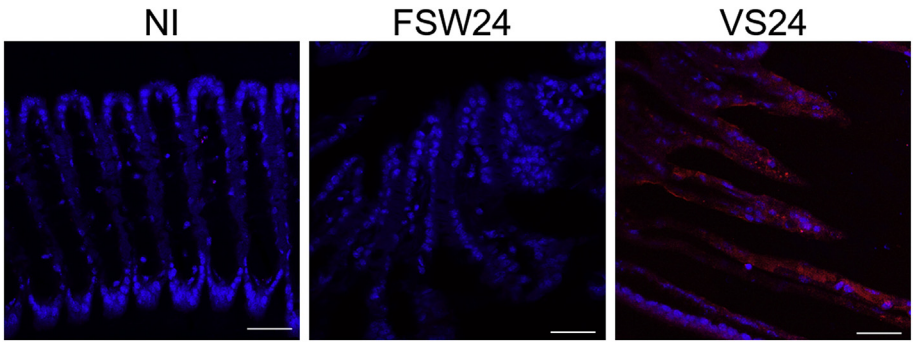
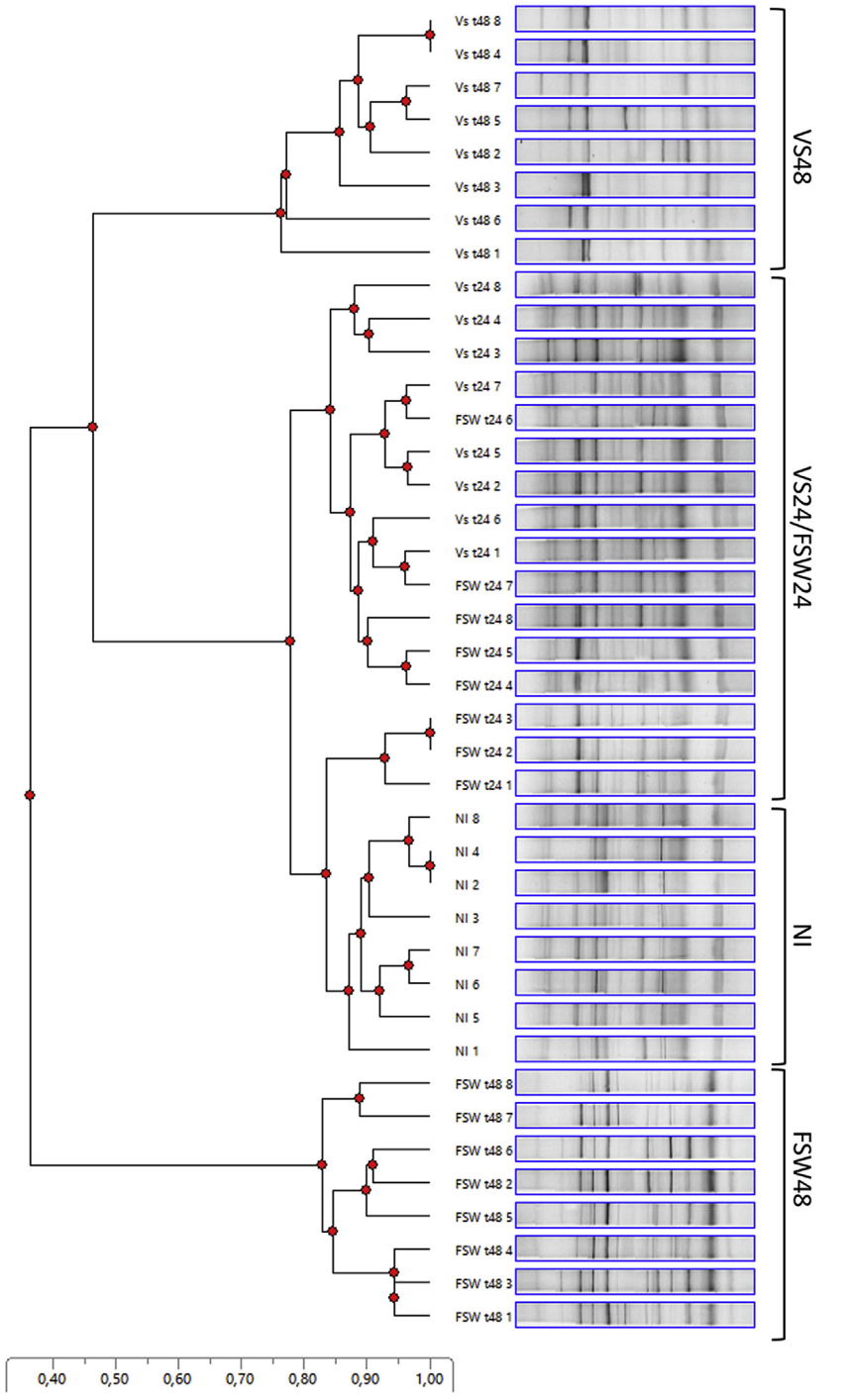


Fig. 2. Detection of the antimicrobial peptide ApBD1 in gill tissues from the scallop *A. purpuratus* after an immune challenge by immunofluorescence and confocal analysis. NI: Non-injected scallops. FSW24: 24h after injection with filtered seawater. VS24: 24h after injection with *Vibrio splendidus*. Alexa 568 goat anti-mouse antibody was used for ApBD1 detection (in red) and TO-PRO®-3 Iodide was used as nucleic acid stain (in blue). Magnification 400X, scale bar, 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



	NI	FSW24	VS24	FSW48	VS48
Richness	17.38 ± 1.30	**13.38± 0.91 ***	16.00 ± 0.92	18.63 ± 1.30	**14.38 ± 0.91 ***
Shannon (H´)	3.83 ± 0.13	3.67 ± 0.09	**3.21 ± 0.12 ***	3.76 ± 0.14	**3.53 ± 0.11 ***

Fig. 3. Venn diagram representing the unique and shared taxa of the microbial community of *Argopecten purpuratus*. Taxa were all present in at least one group with a relative abundance of 0.1%. NI: Non injected; VS24/VS48: 24 h/48h after injection with heat-killed *Vibrio splendidus*; FSW24/FSW48: 24 h/48h after injection with filtered seawater.

based on the obtained rarefaction curves, sequencing depth was sufficient to identify all unique OTUs in the experimental groups (Fig. 1S, A). After filtering rare OTUs and Cyanobacteria, 321 taxonomic groups were identified with 99% common sequence similarity, where 51 belonged to the *A. purpuratus* core microbiota as they were present in the five experimental groups (Fig. 4). The 51 taxonomic groups were identified to the deepest taxonomic group and their relative abundance in each experimental condition was determined (Table 1). The scallop core microbiota was almost exclusively composed by the phyla Bacteroidetes, Chlamydiae, Firmicutes, GN02, OD1, Proteobacteria, Spirochaetes, Tenericutes, Thermotogae and Verrucomicrobia. Of the 51 taxonomic groups, 12 were identified up to the order level, which showed a remarkably higher relative abundance of Bacteroidales in the NI scallops (28.07) compared to the injected scallops (0.29–1.01). At

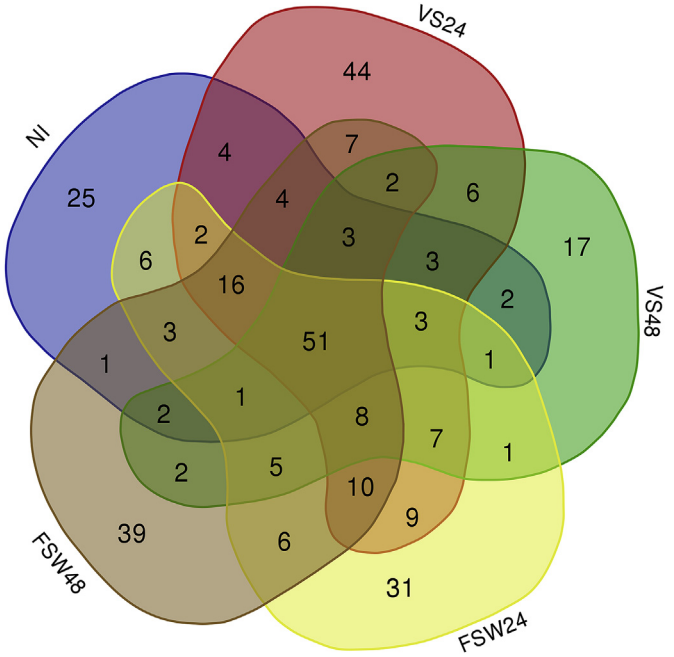


Fig. 4. Relative abundances of bacterial phyla obtained from *Argopecten purpuratus* microbiota exposed to the five experimental conditions. NI: Non-injected; VS24/VS48: 24 h/48h after injection with *Vibrio splendidus*; FSW24/FSW48: 24 h/48h after injection with filtered seawater.

the genus level, the presence of Vibrio in all scallops, showing a slight decrease of its relative abundance in the injected groups (0.48–1.36), was highlighted compared to the NI group (2.45). In turn, Mycoplasma was shown to increase its relative abundance in injected scallops, with higher abundance in the VS group (6.69–6.77) compared to the FSW (3.47–4.55) and NI scallops (1.72) (Table 1).

Regarding the microbiota diversity, results showed that it was higher in VS24 and lower in the NI and VS48 groups, which was determined using the Shannon index (Fig. 1S, B). Similarly, PCoA based on weighted (quantitative) and unweighted (qualitative) UniFrac distances showed that the NI group is the most separated group from the others, and that among injected groups, VS48 has a phylogenetically distinct microbiota community (Fig. S2). Based on weighted UniFrac distances, NI group phylogenetical separation from other groups was evident along PC1 (X axis), and this accounted for 56.6% of data phylogenetic variation (**PC1**, Fig. S2A); however, when based on unweighted UniFrac, the NI group separates from other groups throughout PC2 (Y axis), accounting for 20.9% of dataset phylogenetic distances (**PC2**, Fig. S2B).

OTUs were assigned to 30 phyla (Fig. 5), and in the NI group 83.7% of the community was made up of four dominant phyla: Bacteroidetes (43.8%), Proteobacteria (18.8%), Firmicutes (18.2%) and Tenericutes (2.9%). After scallop injection (FSW or VS), the relative abundance of Bacteroidetes decreased (less than 14% in all groups). Firmicutes became the most abundant phyla, increasing their relative abundance in the injected scallops. Relative abundance of Proteobacteria, Tenericutes and OTUs assigned as "unclassified" increased in all injected groups. These changes were also highlighted in terms of relative abundance of different Classes (Fig. 3S) and Orders (Fig. 6). Compared with the NI group, the injection of *A. purpuratus* with either *V. splendidus* (VS24/VS48) or filtered sea water (FSW24/FSW48) resulted in a noticeable reduction of Bacteroidales and an increase in Mycoplasmatales, Clostridiales and Chlamydiales, among others (Fig. 6). After 24 h, the effect of injecting *V. splendidus* was indicated mainly by a decrease in unidentified orders (Flavobacteriales and Chlamydiales), and an increase in Mycoplasmatales, MBA08 and Rickettsiales (Fig. 6, **FSW24/VS24**). After 48 h, the effect of *Vibrio* injection on *A. purpuratus* microbiota

Table 1
Argopecten purpuratus core microbiota identified to the deepest taxonomical group, and the relative abundance (blue-lower, red-higher) in the five experimental groups: non-injected (NI), 24 h/48h after injection with heat-killed *Vibrio splendidus* (VS24/VS48) and 24 h/48h after injection with filtered seawater (FSW24/FSW48).

		NI	VS24	VS48	FSW24	FSW48
Species	*Lactobacillus helveticus*	0.42	0.41	0.49	0.34	0.27
	Prevotella copri	0.27	0.91	1.09	1.45	0.55
Genus	*Vibrio*	2.45	0.84	1.36	0.48	0.56
	Prevotella	2.25	1.64	1.02	2.14	1.82
	Mycoplasma	1.72	6.77	6.69	3.47	4.55
	Lactobacillus	1.35	1.92	2.99	1.74	2.23
	Enterococcus	0.88	0.79	1.37	1.47	1.25
	Bacteroides	0.66	0.58	1.32	0.46	0.47
	Treponema	0.60	0.60	0.34	0.29	0.53
	Clostridium	0.59	0.66	1.29	0.40	0.34
	Ruminococcus	0.55	0.67	0.56	0.16	0.34
	S1	0.52	1.56	1.01	0.83	1.25
	Syntrophomonas	0.44	0.38	0.58	0.84	0.64
	Phascolarctobacterium	0.39	0.27	0.48	0.19	0.34
	Campylobacter	0.34	0.13	0.45	0.23	0.15
	RFN20	0.32	0.47	0.51	0.55	0.66
	Paludibacter	0.30	0.16	0.34	0.67	0.52
	Winogradskyella	0.30	0.05	0.45	0.16	0.20
	Methanoculleus	0.29	0.21	0.31	0.26	0.17
	Anaerovibrio	0.18	0.39	0.52	0.07	0.35
	Pseudoalteromonas	0.16	0.22	0.48	0.32	0.42
	Oscillospira	0.16	0.57	0.27	0.29	0.14
	Tepidimicrobium	0.14	0.13	1.01	0.17	0.26
	Dialister	0.10	0.35	0.35	0.35	0.29
Family	Campylobacteraceae	4.68	5.87	5.92	5.25	6.45
	Ruminococcaceae	0.84	0.55	0.57	0.63	0.90
	Bacteroidaceae	0.60	0.36	0.38	0.26	0.51
	Brachyspiraceae	0.29	0.47	0.85	0.40	0.42
	Porphyromonadaceae	0.22	0.46	1.42	0.75	0.55
	Mycoplasmataceae	0.22	0.27	0.36	0.40	0.36
	JTB36	0.22	0.41	0.27	0.20	0.28
Order	Bacteroidales	28.07	0.55	0.29	0.55	1.01
	Bacteroidales	7.77	3.21	2.93	2.45	2.65
	Campylobacterales	3.25	4.21	4.17	3.75	4.43
	Clostridiales	1.07	2.61	1.05	2.75	2.84
	Clostridiales	1.02	1.11	1.33	2.56	1.62
	ML615J-28	0.78	0.18	0.63	0.72	0.91
	Chlamydiales	0.68	1.82	2.93	3.55	2.25
	MBA08	0.67	1.03	0.63	0.15	0.52
	Campylobacterales	0.43	0.56	0.48	0.59	0.56
	SHA-98	0.35	0.22	0.96	0.39	0.60
	OPB54	0.21	0.34	0.48	0.26	0.32
	Flavobacteriales	0.16	0.18	0.82	0.26	0.34
Class	Alphaproteobacteria	0.84	2.55	2.35	1.70	1.46
	BD1-5	0.62	0.20	0.49	0.03	0.44
	Gammaproteobacteria	0.51	0.92	1.35	1.06	1.58
	Opitutae	0.38	1.81	0.77	0.92	1.17
Phylum	Bacteroidetes	1.57	0.31	0.66	0.22	0.38
	GN02	0.45	0.66	0.54	0.69	0.38
	OD1	0.15	0.42	0.52	0.29	0.11
Unclassified	Unclassified Bacteria	10.12	17.15	19.90	25.96	19.49

when compared with FSW injection was marked by an increase in Halanaerobiales, Vibrionales and Pseudomonadales, and a decrease in Clostridiales (Fig. 6, **FSW48/VS48**).

The overall modulation in scallop microbiota was then assessed through detection of changes in the functionality of the microbial community predicted by PICRUST analysis. KEGG orthologs were classified to level 3; apoptosis, bacterial invasion of epithelial cells and basal transcription factors were among the most significantly modulated pathways in response to the injection, increasing significantly in both VS groups ($P < 0.05$) (Fig. 4S). The highest relative frequency of the apoptosis pathway was found in both VS24 and VS48, whereas the bacterial invasion of the epithelial cells pathway increased with time in the VS groups, displaying the highest frequency at VS48. FSW groups showed a slight increase in the bacterial invasion pathway compared with the NI group, but not to the extent of the VS groups. The endocytosis pathway showed a different dynamic related to the experimental condition. In the FSW groups, the highest relative frequency was at 24 h, while in the VS groups it was at 48 h compared with the NI group. The signaling pathways mTOR and p53 also increased specifically in the VS groups, except for a slight increase in the p53 signaling pathway in FSW48 (Fig. 4S).

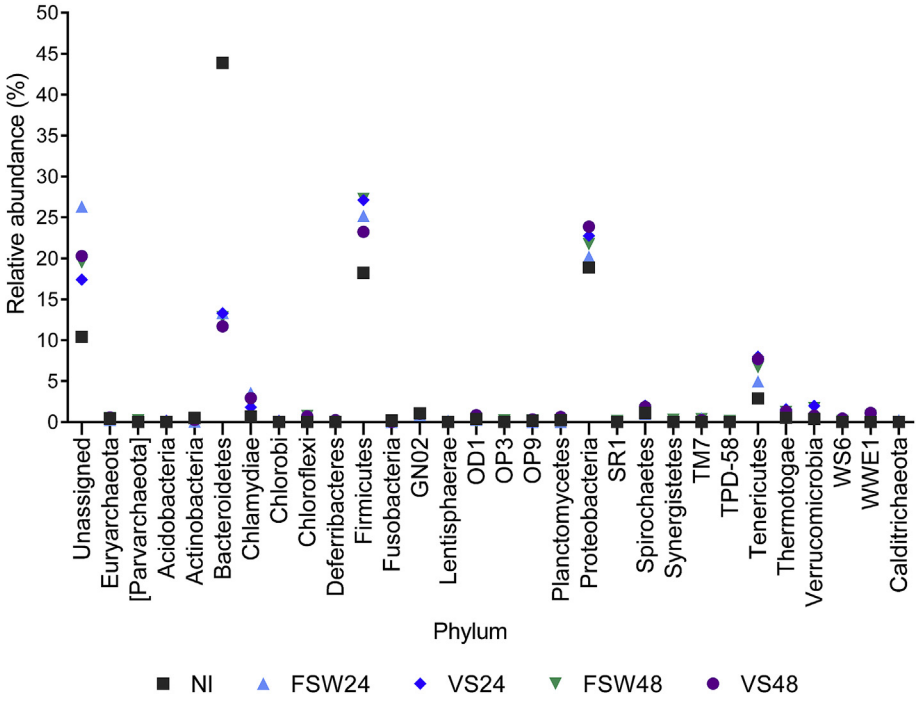


Fig. 5. Relative frequency of the 15 most significant taxonomic orders of the microbial community from *Argopecten purpuratus* under different immune conditions. All pairwise comparisons are significantly different (NI: Non-injected; VS24/VS48: 24 h/48h after injection with *Vibrio splendidus*; FSW24/FSW48: 24 h/48h after injection with filtered seawater; Fisher's exact test, Benjamini-Hochberg FDR $P < 0.05$.

4. Discussion

Disturbance in the balance of the host microbiota can lead to changes in the diversity and abundance of certain bacteria, resulting in beneficial or harmful effects to the host [42]. In this context, the present study showed that the stimulation of *Argopecten purpuratus*, either by injury stress (FSW injection) or bacterial exposure (VS injection), resulted in specific changes in the diversity, abundance and composition of the microbiota according to scallop immune status. All scallops were held together during the *in situ* challenge; the NI group was sampled at the end of the experiment to avoid any microbiota changes related to temporal/spatial variability. Notably, immune stimulation was performed with a heat-killed *Vibrio,* and therefore, the pathogen-microbe interaction effect on the microbiota shift is unlikely. Thus, activation of the scallop immune response might contribute to the modulation of the microbiota, and the host factors that participate in this balance must be characterized.

To assess activation of the scallop immune response after the *in situ* challenge, 11 genes were chosen as potential immune response markers [8]. Remarkably, five significant overexpressed genes were specifically upregulated in the VS group, highlighting a lack of injury stress response genes in the chosen panel. Two of the evaluated genes (*ApBD1* and *Glys*) were overexpressed in both tissues, while the other three genes were overexpressed only in hemocytes (*HSP70* and *PRX*) or gills (*eSOD*). This tissue-specific expression profile could be related to the immune role of hemocytes in the systemic defense versus the local defense provided by gills. Indeed, strong differences in immune gene expression between these tissues has been recently described in *A. purpuratus* [43], suggesting they could display specific roles in host defense.

Overexpression at transcript and protein levels of the AMP ApBD1 was detected in *Vibrio* challenged scallops, which was consistent with previous findings in A. *purpuratus* [11,12] and other immune challenged bivalves [39–41]. Big defensins are a diverse AMP family found in several mollusk species that contribute to host defense against pathogens [44-46]. Interestingly, this effector could play different roles in host homeostasis. ApBD1could display a protective role in the epithelial immune defense of the scallop [47], and it could also participate in the regulation of commensal bacteria, as described for enteric defensins in vertebrates [48,49]. Indeed, the peak of expression of the significant overexpressed immune effectors was detected at 24 h post challenge, and the significant decrease of bacterial diversity and richness was detected at 48 h post challenge. Thus, the expression of antimicrobial effectors such as ApBD1 may participate in the modulation of bacterial composition and abundance. Further research using gene silencing with RNA interference, for example, will help answer this issue.

Two different approaches were used in this study to characterize the microbiota dynamics; both strategies were thought to be complementary. On the one hand, DGGE unveiled the individual diversity variability of the bacterial groups between individual scallops, and also revealed specific changes in the major bacterial community structure at 48 h after injection of FSW or VS. On the other hand, 16S rDNA deep amplicon sequencing helped reach a reliable resolution in taxonomic assignment and identification. Indeed, the plateau reached by the rarefaction curves from the deep sequencing data indicates that bacterial richness from every experimental condition was fully obtained. Moreover, this data allowed for the establishment of a core scallop microbiota among experimental conditions. The major phyla identified in *A. purpuratus* (Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes) have also been detected as major groups in other marine bivalves, such as the oyster *Crassostrea sikamea* [50]. In addition, it is known that the bacterial communities of bivalves such as oysters and mussels are significantly different from those of seawater [51]. Although more studies are needed to declare the existence of a core scallop microbiota, it is tempting to speculate that certain phyla might be constantly colonizing scallop tissues; consequently, they must hold a key role in host homeostasis, as shown earlier in vertebrates [52] and invertebrates [53].

The identification of a core microbiota among the experimental conditions allowed for the identification of changes in the relative abundance of several taxonomic groups between NI and injected scallops, and in some cases between FSW and VS injected scallops. For instance, members of the genus Lactobacillus – known for their probiotic properties [54] – were found in higher abundance in injected groups when compared to non-injected groups. This example modulation might be part of a defense mechanism to increase response capacity and resistance against an infection [55]. As another example, the same modulation was observed in the Proteobacteria genus

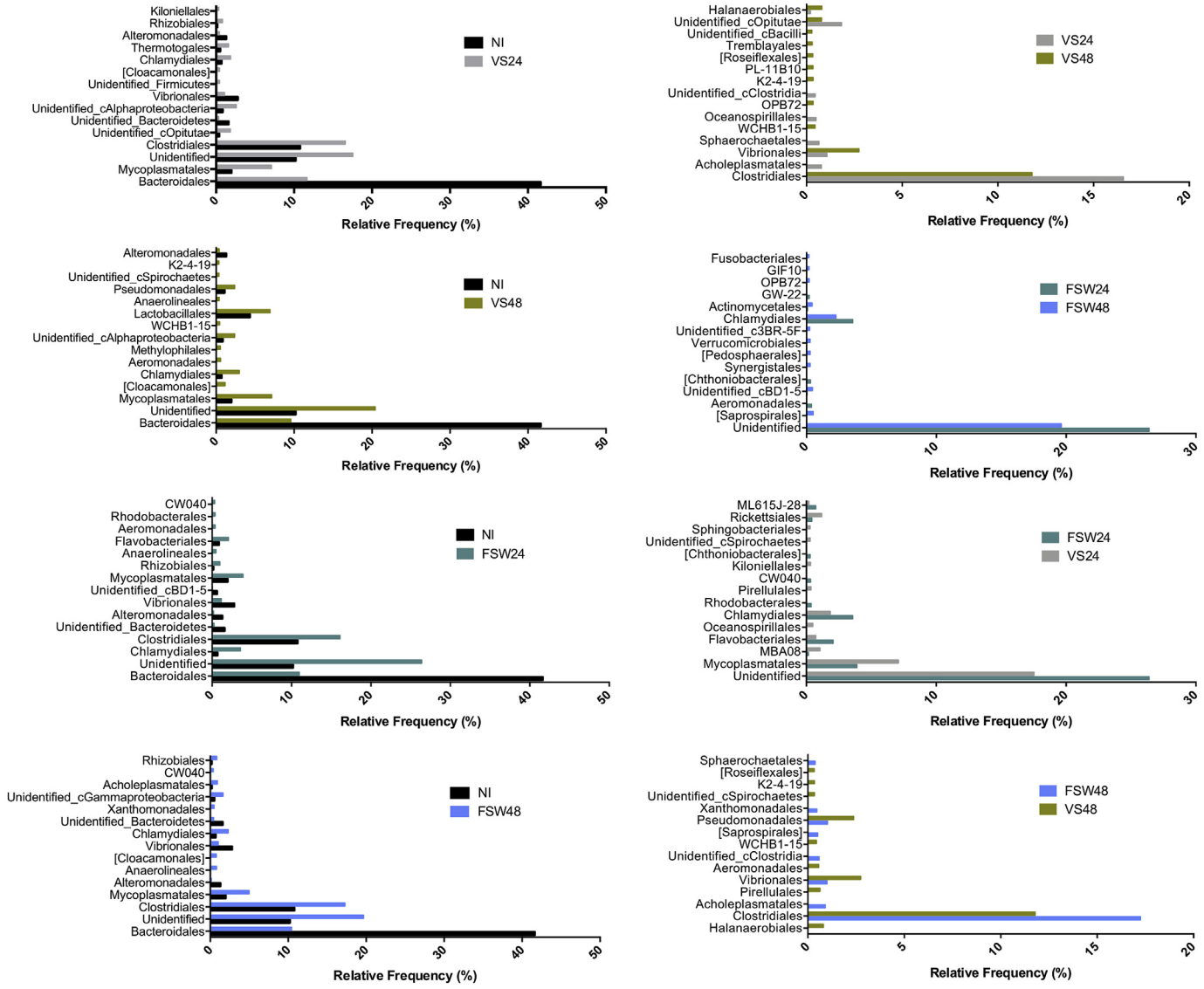


Fig. 6. Dendrogram analysis of DGGE profiles of the predominant bacterial population from *Argopecten purpuratus* exposed to the five experimental conditions. (NI: Non-injected; VS24/VS48: 24 h/48h after injection with *Vibrio splendidus*; FSW24/FSW48: 24 h/48h after injection with filtered seawater. Dendrogram construction, species richness and Shannon H′ index were obtained by CLIQS analysis. Asterisks indicate significant differences ($P < 0.05$).

Pseudoalteromonas, which has been shown to confer protection and enhance survival of several bivalve species [56]. Moreover, this genus is known to produce a wide variety of biologically active secondary metabolites such as broad-spectrum antibiotics and proteases that reduce biofouling [57]. These characteristics might interfere with or regulate the abundance of another species. Besides shifts in the abundance of the core groups, the total loss of certain bacterial taxa was also evident in injected scallops. This loss could have a negative impact on scallop homeostasis, since lower microbiota diversity in invertebrates has been related to the presence of pathogens, and poor species richness has been related to higher susceptibility to pathogen invasion [35]. Scallop experimental challenges with alive pathogenic bacteria are needed in order to address this issue, and to help uncover the complexity of microbiome-host crosstalk during infections.

Remarkably, differential changes in various bacterial groups were identified between the VS and FSW groups, both at 24 and 48 h. For instance, the VS group showed an increase in the orders Halanaerobiales, Vibrionales and Pseudomonadales, and a decrease in Clostridiales compared with the FSW group at 48 h. Members of the genus *Mycoplasma,* for example, were found to increase abundance in response to injection; however, this modulation was potentiated when the injection included *V. splendidus*. These parasites have been found in several bivalve species [58]. Their abundance increase has not only been associated with infections but also during probiotic treatment of *Haliotis gigantea* with *Pediococcus* sp. Ab1 (10 fold increase) [59]. Thus, different bacterial community structures are found associated to scallops according to the immune stimulus. This result highlights the complex bacterial population dynamics that might exist in aquatic organisms, and suggests the possible participation of the host response. The premise of specific changes of the microbiota according to scallop immune status is reinforced by KEGG analysis. The functional prediction identified that several pathways, such as apoptosis, bacterial invasion of epithelial cells and basal transcription factors, increased in response to both types of stimuli, but to a greater extent in the *Vibrio*-challenged scallops. Apoptosis has a key role in immune system homeostasis, function and defense against pathogens, which can be associated with the increase of basal transcription factors to produce more proteins such as immune effectors [60]. In the same context, the endocytic pathway was increased in FSW24 and in VS48, suggesting there are different temporal responses according to the stimulus. The mTOR pathway, which is related to the activation of protein synthesis, lysosome stabilization and autophagy inhibition in mussels [61], and

the p53 signaling pathway, which is related to DNA protection and is a critical regulator of cell cycle response to stress [62], are increased in VS24 and VS48 but only slightly in FSW at 48h, suggesting a differential response according to the stimulus.

Overall, the results obtained from the microbiota analysis of immune challenged scallops in field showed that the diversity, abundance and composition of scallop microbiota differ according to the immune status of scallops. Further elucidation of the link between the immune response–microbiota community structure and host immune capacity may provide important insights on the role of microbiota composition on the health and homeostasis of scallops.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.028.

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