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# Seaweeds influence oyster microbiota and disease susceptibility

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## ABSTRACT

- 1- A growing awareness of role that microbiota can play in mediating the effects of pathogens on hosts has given rise to the concept of the pathobiome. Recently, we demonstrated that the Pacific oyster mortality syndrome affecting *Crassostrea gigas* oysters is caused by infection with the Ostreid herpesvirus type 1 (OshV-1) followed by infection with multiple bacterial taxa.
- 2- Here we extend the concept of this pathobiome beyond the host species and its bacterial microbiota by investigating how seaweed living in association with oysters influences their response to the disease. We hypothesized that by their mere presence in the environment, different species of seaweeds can positively or negatively influence the risk of disease in oysters by shaping their bacterial microbiota and their immune response. Although seaweed and oysters do not have direct ecological interactions, they are connected by seawater and likely share microbes.
- 3- To test our hypothesis, oysters were acclimated with green, brown or red algae for 2 weeks and then challenged with OshV-1. We monitored host survival and pathogen proliferation and performed bacterial microbiota and transcriptome analyses.
- 4- We found that seaweeds can alter the bacterial microbiota of the host and its response to the disease. More particularly, green algae belonging to the genus *Ulva* spp. induced bacterial microbiota dysbiosis in oyster and modification of its transcriptional immune response leading to increased susceptibility to the disease.
- 5- This work provides a better understanding of a marine disease and highlights the importance of considering both macrobiotic and microbiotic interactions for conservation, management and exploitation of marine ecosystems and resources.

## KEYWORDS

bivalve, disease ecology, epidemiology, macroalgae, microbiome, pathogen

## 1- INTRODUCTION

Pathology has traditionally focused on single host–single pathogen interactions under given environmental conditions (Ben-Horin et al., 2015; Cory & Deschodt, 2017; Vayssier-Taussat et al., 2014). However, invertebrate host and their pathogens are rarely found in isolation in natural situations. They indeed both exist within diverse and complex ecological communities, and interactions can influence the transmission and the impact of pathogens. Even where a primary agent can be identified, its effect is often influenced by other species interacting with the host, the pathogen or both (Bass et al., 2019; Byers, 2020; Sweet & Bulling, 2017; Vayssier-Taussat et al., 2014). More particularly, the microbiome, broadly defined as a collection of micro-organisms living with the host (including bacteria, archaea, viruses, fungi and unicellular eukaryotes), is increasingly recognized as a factor modifying interactions with pathogens. The pathobiome concept breaks down the idea of one pathogen = one disease, and highlights the role of the microbiome in pathogenesis (Bass et al., 2019; Sweet & Bulling, 2017; Vayssier-Taussat et al., 2014).

In aquatic ecosystems, micro-organisms from one host species can be released into the water column, transported by currents and thus colonize neighbouring hosts of various species. Thus, all hosts in the same system can exchange micro-organisms horizontally via the water column, and the concept of pathobiome can be extended to all ecosystem components including the sediment (Bass et al., 2019). Disruption of the microbial profile within the host caused by pathogen infection, physiological status, population density or environmental perturbations can potentially lead to a pathobiot

A well-known case which illustrates the concept of pathobiome is the Pacific oyster mortality syndrome (POMS). POMS emerged in 2008, causing massive mortality in juvenile oysters, and rapidly became panzootic (Pernet et al., 2016). Infection by Ostreid Herpesvirus type 1  $\mu$ Var (OsHV-1  $\mu$ var) is the first critical step in the infectious process, leading to an immunocompromised state by altering haemocyte physiology (de Lorgeril et al., 2018). The viral infection is followed by dysbiosis, an imbalance in the host microbial community that is associated with disease. Dysbiosis leads to a secondary colonization by opportunistic bacterial pathogens and oyster death. The reshuffling of the oyster microbiota is an integral part of the infectious process induced by OsHV-1 (Lucasson et al., 2020). In addition, oysters with differing levels of resistance to POMS exhibit constitutive differences in their bacterial microbiota, suggesting that it might play a role in host immunity (Clerissi et al., 2020; King et al., 2019; Pathirana et al., 2019). More broadly, the oyster microbiome varies according to tissues, geographical location, food regime, temperature, infection or antibiotic use (Clerissi et al., 2020; Dupont et al., 2020; King et al., 2019; Lokmer et al., 2016; Lokmer & Wegner, 2015; Wegner et al., 2013)

The risk of OsHV-1 mortality differs depending on the species that live around the oysters (Pernet et al., 2014; Pernet et al., 2021). Mortality risk of oysters held in mussel farms is lower than in oyster farms or empty farms, suggesting that mussels reduce the infection pressure on the host (Pernet et al., 2014). It is also possible that species that live around the oysters harbour species-specific bacterial microflora that influence the host microbiota, its immune response and its susceptibility to POMS (Pernet et al., 2021). The oyster pathobiome may be extended beyond the host species and its microbiota, leading to community models in which co-occurring species influences the health of the host population.

In this paper, we hypothesized that by their mere presence in the environment, different species of seaweeds can positively or negatively influence the risk of disease in oysters by shaping their bacterial microbiota and their immune response. Although seaweed and oysters do not have direct ecological interactions such as competitive, predatory or symbiotic relationships, they are connected by seawater and likely share microbes. Seaweed indeed harbour a rich diversity of associated bacteria (Egan et al., 2013) and their presence influence disease resistance of co-cultured shrimps (Anaya-Rosas et al., 2019). Here we developed a general study framework which include the oyster, its pathogen and bacterial microbiota, and seaweeds that can be applied to any other aquatic host.

To test our hypothesis, we exposed oysters to green *Ulva* spp., brown *Fucus vesiculosus* or red algae *Solieria chordalis* under laboratory conditions for 2 weeks and challenged them with OsHV-1. We chose these seaweeds because they thrive in different habitats and their bacterial microbiota was expected to be different (Lachnit et al., 2011; Singh & Reddy, 2014). We monitored host survival and pathogen proliferation and we conducted bacterial microbiota and transcriptome analyses.

## 2- MATERIALS AND METHODS

### 2-1 Living organisms

Specific Pathogen-Free (SPF) Pacific oysters *Crassostrea gigas* were produced under controlled conditions (Le Roux et al., 2016; Petton, Boudry, et al., 2015). Briefly, adult oysters were collected in the wild in Fouras (Marennes-Oléron, France; 46°0'43"N, 1°7'3"W) and moved to a grow-out farm located at Aber Benoît (northern Brittany, France; 48°34'30"N, 4°36'18"W) until being used as broodstock. In January 2018, they were transferred at the Ifremer marine station located at Argenton (Brittany, France; 48°31'16"N, 4°46'2"W, France) in 500-L flow-through tanks at 17°C and fed ad libitum for 6 weeks for conditioning. Gametes from 76 females and 12 males were collected by stripping and fertilized on 20 February 2018 as previously described (Petton, Boudry, et al., 2015). The oyster larvae were raised for 15 days at 25°C in 5-L cylindrical tubes and settled on clutch for 10 days. The resulting spat were then transferred on 5 April 2018, at the Ifremer marine station located at Bouin (France) for nursery. They were then transferred back to the Ifremer facilities at the Argenton experimental site at 4 months old, for the start of the experiment on 6 July 2018. The oysters were screened using an OsHV-1-specific quantitative PCR assay at the different stages of production and no OsHV-1 DNA was detected.

Before the experiment, oysters were maintained at 21°C in 500 L flow-through tanks, supplied with seawater filtered to 5  $\mu$ m and exposed to UV irradiation. Animals were fed constantly with a mixture of two phytoplankton species, the diatom *Chaetoceros muelleri* (CCAP 1010/3) and the Prymnesiophyceae *Tisochrysis lutea* (CCAP 927/14; 1:1 in dry weight). Food concentration was measured daily using an electronic particle counter (Coulter Multisizer 3) equipped with a 100  $\mu$ m aperture tube and maintained at c. 1,500  $\mu$ m<sup>3</sup> / $\mu$ l in seawater at the outlet of the tanks. Temperature (21°C), salinity (34–35 g/L), pH<sub>NBS</sub> (c. 8.0) and oxygen (c. 100%) were daily controlled with the WTW probes xi3101, cond340, pH 3310 and FDO 925, respectively (Fisher scientific, Illkirch-Graffenstaden, France).

Seaweeds were collected on 5 July 2018 in the bay of Brest at Anse du Roz (48°19'21.7"N, 4°19'07.9"W) while seawater temperature was c. 15.0°C. We retrieved the green algae *Ulva* spp. (Ulvophyceae), the brown algae *Fucus vesiculosus* (Phaeophyceae) and the red algae *Solieria chordalis* (Rhodophyceae). While *F. vesiculosus* and *S. chordalis* are easily visually identifiable species, this is not the case for *Ulva* spp. which exhibit multiple phenotypes and require DNA sequencing. As we have not performed any molecular analysis, identification of *Ulva* is limited to genus. These seaweeds were chosen because they thrive in different habitats, more or less anthropized, and their bacterial microbiota was expected to be different (Lachnit et al., 2011; Singh & Reddy, 2014). The green and the brown algae thrive in the middle shore of the intertidal zone and were collected by foot while the red algae are from the upper sublittoral level and were collected by scuba diving. The proliferation of green algae is generally associated with eutrophication, that is, nutrient enrichment beyond the selfregulatory capacity of the marine ecosystem. In Brittany, since the 1970s, green algae outbreak every year late spring or early summer (Morand & Merceron, 2005). The red algae *S. chordalis* is often found in healthy sheltered areas in calm waters, attached to a variety of substrates including gravel, shells and other algae. In the bay of Brest, *S. chordalis* is found in locally protected areas and in association with maërl bed (Floc'h et al., 1987). Finally, *Fucus vesiculosus* naturally live in the zone of high water of neap tides (Hily & Jean, 1997). Green and brown algae species naturally coexist with oysters, while the red algae species is found nearby in deeper areas.

Seaweeds were collected attached to their substrata (stones, shells or pieces of maërl) and maintained alive during the entire duration of the experiment. Once in the laboratory, each group of seaweed and their associated micro-organisms were kept separated in three 500 L flow-through tanks after rinsing with seawater, until the onset of the experiment. Our study did not require ethical approval.

## 2-2 Experimental design

Here we used an open-flow system developed in a previous study (Pernet et al., 2021; Figure 1a). Virus-contaminated seawater enriched with phytoplankton was evenly distributed to 12 experimental units, each consisting of one header tank containing the seaweeds (green, brown or red algae) or left empty as control connected to one holding tank containing the SPF oysters (Figure 1a). Each experimental unit was run in triplicate. Each header tank was connected to the seawater supply, to the food supply and to the source of infection by flexible tubes fitted inside a peristaltic pump. The seawater flow was set at 300 ml/min at the entry of each header tank. Seawater overflowed from the header tank to the recipient tank. All the tanks were 45 L (60 × 40 × 19 cm) and illuminated by LED (EHEIM powerLED+) covering the full spectrum of sunlight to support the growth of algae. The photoperiod was fixed at 14L/10D. Seawater was homogenized by means of a recirculation pump and air bubbling to maintain O<sub>2</sub> level in all tanks. The phytoplankton concentration was adjusted at the inlet of the header tank containing the seaweed (or control) to obtain ~800 μm<sup>3</sup> /μl at the outlet of the holding tank (level 3). Seawater was sampled daily at the inlet and outlet of each holding tank to determine phytoplankton consumption using the electronic particle counter. The experiment consisted of two successive phases: acclimation and viral exposure (Figure 1b).

### 2-2-1 Acclimation phase

Seaweed and recipient oysters were placed in header tanks and holding tanks, respectively, on 6th July 2018 (Figure 1). The seaweeds were well interspersed throughout the available volume of the tank. We put equivalent volume of algae for each species, corresponding to about 600 g wet weight of brown and green algae, and 1,000 g wet weight of red algae. The biomass of oysters was c. 170 g ± 0.1 in each tank, corresponding to 340 individuals. Acclimation lasted for 14 days until 20 July 2018.

### 2-2-2 Viral exposure

The SPF oysters for injection were myorelaxed in MgCl<sub>2</sub> (50 g/L in a mixture of seawater and distilled water 40/60 v/v) at 21°C (Suquet et al., 2009) on 19th July 2018. A total of c. 600 oysters (2.13 kg) were individually injected in the adductor muscle with 50 μl of viral suspension, containing 1.7 × 10<sup>5</sup> copies of OsHV-1 μVar/μl diluted by 10× (Schikorski et al., 2011). They were kept without feeding in a 45 L tank in static oxygenized seawater for 24 h where they shed viral particles (Figure 1). At this point, the seawater surrounding the donors became contaminated with the virus and used as the source of infection connected to the seawater distribution network by flexible tubes fitted inside a peristaltic pump (Pernet et al., 2021). Infection therefore began on day 14 of the experiment (Figure 1b). The water input from the source of infection was arrested after 7 days of exposure. The donors were then removed. Survival of oysters placed in the virus-contaminated seawater (donors) and in the recipient tanks (level 3) was monitored daily for 16–20 days, and dead animals were removed at the end.

Due to logistical constraints, there was no uninfected control. In our experimental conditions, the survival of these controls is always 100% so that they are generally excluded from the survival analyses (see Pernet et al., 2021 and references therein). Therefore, we cannot decipher the effect of virus from that of time (and any potential confounding time-related effects) during the viral exposure phase.

## 2-3 Sampling

Oysters (*N* = 5 individuals) were sampled and weighted during acclimation at day 5, 12 and 14, and after viral exposure 72 h postinfection (hpi, 17 days) in each recipient tank (Figure 1b). Soft tissues were removed from the shells, pooled together and flash frozen in liquid nitrogen and stored at –80°C. Samples were then ground in liquid nitrogen with a MM400 homogenizer and the resulting powder was subsampled for bacterial microbiota (metabarcoding), gene expression (RNA sequencing) and lipid (classes and fatty acid). Five oysters were also individually sampled in each tank 72 hpi and directly ground for OsHV-1 DNA detection. Tissues were immediately homogenized with a polytron homogenizer in 2 ml of sterile artificial seawater and stored at –20°C until analysis.

Seaweeds (2 g) were sampled during acclimation at days 6, 11 and 14 for metabarcoding analyses, and at day 14 for fatty acid analyses (Figure 1b). As for oysters, seaweed samples were flash frozen in liquid nitrogen, stored at –80°C and ground in liquid nitrogen.

Seawater (c. 1 ml) was sampled at 72 hpi at the inlet and outlet of the tanks containing the oysters for OsHV-1 DNA qPCR analyses (Figure 1b). Seawater was stored in sterile 1.5 ml Eppendorf tubes at –20°C until analysis.

## 2-4 Analyses

### 2-4-1 Quantification of OsHV-1

The detection and quantification of OsHV-1 DNA was carried out on seawater samples and individual oysters at 72 hpi, using a previously published qPCR protocol (Taqman, Martenot et al., 2010) by Laboceca, a French public diagnostic laboratory (Quimper, France, see File S1)

### 2-4-2 Bacterial microbiota analysis

Analyses were conducted as described in de Lorigeril et al. (2018). Briefly, DNA was extracted from 30 mg of frozen algae powders (sampled at days 6, 11 and 14) and oyster powders (sampled at days 5, 12, 14 and 17) using the DNA from tissue MachereyNagel kit (reference 740952.250). DNA concentration and purity were checked with the Nanodrop ND-1000 spectrometer. For each sample, 16S rDNA amplicon libraries were generated using the 341F-CCTACGGGNGGCWGCAG and 805RGACTACHVGGGTATCTAATCC primers targeting the variable V3V4 loops for bacterial communities (Klindworth et al., 2013). Library were generated using the Illumina two-step PCR protocol and normalized SequelPrep plates. Paired-end sequencing with a 250-bp read length was performed at the Bio-Environment UPVD technology platform (University of Perpignan Via Domitia Perpignan, France) on a MiSeq system (Illumina) using v2 chemistry according to the manufacturer's protocol. The FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented into a galaxy instance was used to define Operational Taxonomic Units (OTU) and to compute taxonomic affiliations (Escudie et al., 2018). Paired reads were merged using FLASH (Magoč & Salzberg, 2011). After denoising and primer/adaptor removal with Cutadapt (Martin, 2011), clustering was performed with SWARM, which uses a clustering algorithm with a threshold (distance = 3) corresponding to the maximum number of differences between two OTUs (Mahé et al., 2014). Chimeras were removed using VSEARCH (Rognes et al., 2016). We excluded chloroplasts from the dataset and filtered out singletons and produced affiliations using Blast against the Silva 16S rDNA database (release 132, December 2017) to produce an OTU and affiliation table in the standard BIOM format. Rarefaction curves of the species richness, confirming a sufficient sequencing, were generated using the Phyloseq R package and the rarefy\_even\_depth and ggrare functions (McMurdie & Holmes, 2013). We used Phyloseq to obtain community composition and to infer diversity metrics at the OTU level using R v3.3.1 [R Development Core Team, R Foundation for Statistical Computing (<http://www.R-project.org>)].

### 2-4-3 Transcriptome analysis

We analysed the whole transcriptome of oysters to compare gene expression after 14 days of acclimation to different seaweeds. RNAs were extracted using 1.5 ml of Extract-All (EuroBio GEXEXT04 OU) from 30 mg of the oyster powders used for bacterial microbiota analysis (see sampling section). RNA concentration and purity were checked using the Nanodrop spectrometer, and their integrity was analysed by capillary electrophoresis on a BioAnalyzer 2100 (Agilent). RNA-seq library construction and sequencing were performed at the Bio-Environment platform. PolyA+ libraries were constructed using the NEBNext Ultra II directional kit according to the manufacturer's instructions from 1 µg of total RNA and sequenced on a NextSeq550 Instrument (SE 75 bp). Data were analysed using a local galaxy instance (Goecks et al., 2010). Reads quality was checked using the Fastq-X toolkit and since all reads display a Phred score above 26 over 90% of their length, no subsequent quality filtering was done (Goecks et al., 2010). Adaptor trimming was then performed using CutAdapt (Martin, 2011). Mapping to the *C. gigas* reference genome [assembly version V9 from (Zhang et al., 2012)] was performed using RNASTAR using default parameters (Galaxy Version 2.4.0d-2 from Dobin et al., 2012). The HTSeq-count tool was used to count the number of reads overlapping annotated genes (Galaxy Version v0.6.1; Anders et al., 2014). Differential gene expression levels were analysed with the DESeq2 R package implemented into a galaxy instance [DESeq {DESeq2} from (Love et al., 2014)]. Fold changes between conditions were considered significant when the adjusted p-value for multiple testing with the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR), was <0.05.

### 2-4-4 Gene ontology annotation analysis

Functional analyses of the differentially expressed genes (DEGs) were performed de novo on assembled transcripts using gene ontology annotation. Blastx comparison against the NR database was performed for the 28,027 genes annotated in the genome, with a maximum number of target hits of 20 and a minimum e-value of 0.001. XML blast result files were loaded onto Blast2GO (Conesa et al., 2005) for GO mapping and annotation with the b2g\_sep13 version of the B2G database. GO terms of differentially expressed genes (DEGs, over- and under-expressed) and associated p-value (DESeq 2) were used as inputs for REVIGO (REduce + VIsualize Gene Ontology), to obtain hierarchical visualization of non-redundant GO term set (Supek et al., 2011). Over-represented GO terms in one condition compared to another were visualized using hierarchical treemaps (biological process) and scatterplots (molecular function).

### 2-4-5 Gross physiology of oysters

Although oysters are filter-feeders and do not directly feed on seaweed, they still might be exposed to dissolved and particulate compounds that potentially alter the response to pathogens. We therefore provide complementary measurements of host growth, food ingestion, energy reserves and trophic indicators to investigate potential confounders.

From daily measurements of phytoplankton concentration at the inlet and outlet of each oyster tank (see previous sections), we calculated the food consumption expressed in volume of microalgae consumed per minute once a day over the duration of the experiment. Daily ingestion rates were averaged over the acclimation periods for each condition and tank.

We evaluate the triacylglycerol to sterol ratio (TAG/ST) of oysters at the end of the acclimation period. The TAG/ST is indeed a proxy of physiological condition in marine organisms associated with disease resistance or tolerance in oysters (Pernet et al., 2019). A subsample of the oyster

powder sampled at 14 days was placed in glass vials containing chloroform–methanol (2:1 v/v) and stored at  $-20^{\circ}\text{C}$  under nitrogen until analyses (File S1).

We investigated with fatty acids whether the seaweed had contributed to the oyster diet. We analysed the fatty acid composition of total lipids in seaweed (food sources) and of neutral lipids in oysters (consumers) as described in file S1. We consider that the fatty acid composition of a consumer reflects that of his food sources ('you are what you eat' principle), and that seaweeds have very different fatty acid profiles depending on the species (Dalsgaard et al., 2003). Therefore, if the oysters have consumed seaweed, this will show in their fatty acid profile.

## 2-5 Statistics

The survival time curves of oysters exposed to OsHV-1 were compared using the Cox regression model considering the effect of seaweed (4 levels, green, brown, red and control condition). Each tank was considered as cluster using the sandwich method to obtain robust parameter estimates. The proportionality of hazards (PH) was checked with martingale residuals.

Mixed-model ANOVAs were performed to assess differences in (1) prevalence and quantification of OsHV-1 DNA in oysters 72 hpi depending on seaweed (4 levels), (2) OsHV-1 DNA in seawater 72 hpi depending on seaweed (4 levels, main plot), levels (2 levels, inlet and outlet of the holding tank, subplots) and their interaction, and (3) oyster total body mass, ingestion rate and physiological condition (triglyceride to sterol ratio) depending on seaweed (4 levels).

The unit of replication (random factor) was the tank in which the seaweed conditions were applied. Tukey's HSD was used as a post-hoc test. The statistical threshold for rejection of the null hypothesis was set at 0.05. When  $0.05 < p < 0.10$ , power analysis was conducted to assess the probability of accepting the null hypothesis when it is false. In general, a power of 0.80 is acceptable. The normality of residuals and homogeneity of variances were graphically checked, and the data were log-transformed where necessary. Prevalence data were fitted a binomial distribution. When OsHV-1 DNA was detected but below the quantification threshold (10 cp/ $\mu\text{l}$ ), individuals were considered positive and assigned the value of 10 cp/mg DNA. All these analyses were conducted with the SAS software package (SAS 9.4; SAS Institute).

Microbial assemblages were compared across tissue types (algae and oysters) and sampling time according to the following procedure using R v3.3.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Principal coordinate analyses (PCoA, {phyloseq}) were computed to represent dissimilarities between samples using the Bray–Curtis distance matrix (ordinate, {phyloseq}). Multivariate homogeneity of group dispersions was tested between bacterial assemblages among samples using 999 permutations (permutest, betadisper, {vegan}). The 200 most abundant OTUs (to the lowest affiliated rank) were selected and used for DESeq2 analyses (DESeq {DESeq2}) to identify those whose abundance varied between conditions (Love et al., 2014). We first identified taxa whose relative abundance changed among conditions after the acclimation period by comparing oysters exposed for 14 days with a specific alga and their controls collected at the same time. Then, we identified taxa (genera) whose relative abundance changed during the infection phase by comparing oysters acclimated for 14 days with a specific alga and their counterparts exposed to the virus collected at 17 days. Heatmaps of taxa (genera) with significant changes in abundances during acclimation and infection phases were computed using the  $\log_2$  of relative abundances and Multiple Array Viewer software (Supek et al., 2011).

## 3 RESULTS

### 3-1 Oyster survival and OsHV-1

Survival of oysters acclimated to green algae and further exposed to OsHV-1 was only  $52 \pm 5\%$ , compared to  $66 \pm 11\%$  for controls,  $72 \pm 17\%$  for brown algae and  $83 \pm 13\%$  for red algae (Figure 2a). Odds of oyster mortality was twice as high in the green algae condition as in the control (Table 1). Conversely, the probability of oyster mortality was half as high in the red algae conditions compared to the control. This result was close to statistical significance and the power value of the test was low (0.523). There was no detectable difference in survival among oysters acclimated to brown algae and controls (Table 1).

The effect of seaweed on viral prevalence in oysters 72 hpi was close to statistical significance ( $p < 0.1$ ), but appeared higher in individuals acclimated to green algae (on all three tanks, 14 out of 15 individuals were positive) than in other conditions (<7 positive out of 15 individuals, Figure 2b; Table S1). This trend was confirmed by higher amounts of viral DNA detected in oysters acclimated to green algae (Table 2). Viral load in seawater at the inlet of the oyster tanks was similar among conditions (between  $9.8 \times 10^3$  and  $1.7 \times 10^4$  OsHV-1 DNA copies/ml, Figure S1).

### 3-2 Bacterial microbiota

We analysed bacterial communities using 16S metabarcoding during acclimation in seaweeds and oysters (5–6, 11–12, 14 days) and infection in oysters (17 days). A total of 9,636,786 reads were obtained from 292 libraries. After the cleaning and filtering steps, 8,330,874 sequences were affiliated to 20,105 OTUs. After removing chloroplasts and non-bacteria, a total of 18,176 OTUs were kept for further analyses (Supplementary data 1; Figure S2). The bacterial microbiota of algae varied among species and remained stable during the acclimation period (Figure 3a and Figure S3, and PERMANOVAs Table S2). Diversity indices in algae did not change over time (Figure S4).

The bacterial microbiota of oysters acclimated to green algae diverged from that of controls after 12 days while it remained similar for other conditions (Figure 3a and Figure S3, PERMANOVA Tables S2 and S3). This was reflected in indices of bacterial diversity which all increased in oysters during acclimation to green algae while they remained stable for other conditions (Figure S5).

During the viral exposure phase, the addition of OsHV-1 induced a shift in the bacterial microbiota of oysters held with brown algae and red algae and resulted in an increase in diversity indices (Figure 3a and Figure S5, PERMANOVA Tables S2 and S3). Overall, in the green algae condition, bacterial microbiota of oysters changed during the acclimation phase, whereas in other conditions, changes occurred later during viral exposure. Class-level assignment of bacterial OTUs indicated that oysters acclimated to green algae showed a marked decrease in the

relative abundance of *Mollicutes* at the benefit of *Alphaproteobacteria* between 5 and 12 days (Figure S3). These changes occurred in oysters held with brown or red algae later during viral exposure (17 days).

In addition, we analysed bacterial communities (200 most abundant OTUs) at the genera level during acclimation to algae and viral exposure. The most significant changes occurred in oysters acclimated to green algae (compared to control at 14 days). The relative abundance of 28 OTUs changed (26 were over-represented, 4 of them belong to *Rhodobacteraceae*) and 20 of them were shared with algae (Figure 3b). There was no difference in the relative abundance of OTUs between oysters acclimated to red algae and control (Figure 3c). In oysters acclimated to brown algae, only three OTUs specific to oysters were modified (Figure 3d)

Viral exposure was associated with significant changes in the relative abundance of OTUs in oysters acclimated to green algae (16 OTUs, 6 over-represented), brown algae (21 OTUs, 15 over-represented) and red algae (22 OTUs, 12 over-represented; Figure 3b–d). Among these OTUs, seven were common to the three conditions. Four genera were over-represented (*Aureispira*, *Poseidonocella*, *Roseovarius* and *Olleya*), whereas the others were under-represented (*Salinirepens*, *Flavobacteriaceae* and *Ulvibacter*). Two of these over-represented OTUs were detected in green algae at the beginning of the acclimation phase (genera *Poseidonocella* and *Olleya*, Figure 3b–d). It is noteworthy that the relative abundance of one OTU corresponding to the genus *Vibrio* increased during infection in oysters acclimated to green algae.

### 3-3 Oyster transcriptomic response

We compared the transcriptomic response of oysters acclimated to green, brown and red algae with that of controls. We sequenced 12 samples that yielded between 25 and 30 million Illumina single-end reads of 75 bp per sample. Between 96.4% and 96.9% of these reads mapped to the *C. gigas* V9 reference genome and were used for identification of differential expressed genes (DEGs). The transcriptome of oysters in contact with algae was modified compared to untreated controls (Figure 4a). We found 311 DEGs (222 overexpressed) in oysters acclimated to green algae versus control oysters, compared to 299 and 128 DEGs in those held with brown and red algae, respectively (167 and 55 overexpressed, respectively, Figure 4a; Supplementary data 2). DEGs were predominantly specific to each seaweed, very few DEGs were shared among all conditions.

Functional analyses of DEGs by condition showed that the defence response was the only category of biological process that was enriched in the three conditions. Defence response category was the second over-represented category in oysters acclimated to green algae, the first over-represented category in oysters acclimated to red algae and was found under 'RNA secondary structure unwinding' category in oysters acclimated to brown algae (Figure S6). As observed for all DEGs, the number of DEGs related to defence response was the highest in oysters acclimated to green algae (32, 22 upregulated), intermediate in oysters held with brown algae (26, 13 upregulated) and the lowest in oysters acclimated to red algae (9, 2 upregulated; Figure 4b). No common DEG for this category was identified between the three conditions. In oyster acclimated to green algae, the DEGs related to defence response were mainly associated with potential immune recognition proteins such as complement C1q, C- and P-lectin (Perlucin and cation-dependent mannose-6-phosphate receptor), toll-like, scavenger receptor (Deleted in malignant brain tumours 1-like) and low affinity immunoglobulin epsilon Fc receptor and to enzymes such as laccase and tyrosinase (Figure 4c; Supplementary data 2). In addition, DEGs related to defence response were mainly associated, in terms of molecular function, to binding function to carbohydrate, calcium ion, metal ion, zinc ion, protein, copper ion, TIR domain, RNA and DNA (Figure S7).

### 3-4 Gross physiology

We measured host growth, food ingestion, energy reserves and trophic indicators to investigate potential confounders. None of these parameters were affected by seaweed (Figure S8). At the onset of the viral exposure, total body mass and energy reserves (lipid, TAG/ST) and food ingestion were similar among conditions. Also, fatty acid compositions of green, brown and red algae were different and did not reflect in the neutral lipid of oysters, suggesting that seaweeds did not contribute to their food regime

## 4 DISCUSSION

Here we showed that different species of seaweeds can positively or negatively influence the risk of disease in oysters by shaping their bacterial microbiota and their immune response. Although seaweed and oysters do not have direct ecological interactions, they are connected by seawater and share microbes. Our study therefore extends the concept of pathobiome beyond recognized pathogens to other ecosystem components, and contributes to establishing a community perspective of host–pathogen interactions (Dittami et al., 2021). These results are therefore important to better understand the risk of disease in marine ecosystems. The study framework developed here can be applied to other analogous systems of interaction than the specific one of algae and oysters, such as those particularly threatened by diseases including corals, amphibians and farmed organisms.

We found that green algae belonging to the genus *Ulva* spp. increased mortality and viral proliferation in the host, induced early dysbiosis of the host's bacterial microbiota and overexpression of genes involved in defence response. Although field studies showed that the proliferation of green algae has dramatic impacts on macrozoobenthic and microbial communities (Quillien et al., 2015; Wang et al., 2020), no studies to date have established a link with the POMS or other marine diseases. Our results suggest that the rising tide of green algal bloom worldwide due to human-induced eutrophication (Smetacek & Zingone, 2013; Ye et al., 2011) may have consequences on oyster–pathogen interactions and disease risk. More broadly, our results are in agreement with the fact that environmental and human-mediated disturbances of natural ecosystems can shape bacterial pathobiomes and promote the emergence of diseases (Vaussier-Taussat et al., 2014). For instance, invasive exotic annual grasses can indirectly increase barley and cereal yellow dwarf viral disease incidence in California native perennial bunchgrasses (Malmstrom et al., 2005).

Our results on oysters differ from those obtained on the shrimp *Litopenaeus vannamei*. Indeed, the green algae *Ulva lactuca* positively influence their microbiota (Mangott et al., 2020) and improve their resistance to bacterial and viral diseases through immune and antioxidant

responses (Anaya-Rosas et al., 2019). This discrepancy, which possibly reflect specific differences between host organisms and their immune defence mechanisms, underscores the importance of developing this study framework on other species.

Dysbiosis in oysters acclimated to green algae *Ulva* spp. consisted of an increase in bacterial diversity, a decrease relative abundance of bacteria from the class *Mollicutes* at the benefit of *Alphaproteobacteria*, and over-representation of the family *Rhodobacteraceae*. Although bacterial diversity is often associated with better health of the host community, this is not always the case (Bass et al., 2019). Tissue damage from infections can promote colonization by a wide variety of opportunistic bacteria from the surrounding environment. This is what happens during OsHV-1 infection (see de Lorgeril et al., 2018; Lucasson et al., 2020 and this study), and in other diseases affecting corals (Closek et al., 2014; Roder et al., 2014). The shift in the relative abundance of *Mollicutes* at the benefit of *Alphaproteobacteria* has never been reported before. In contrast, *Mollicutes* can be more abundant in diseased oysters than in healthy ones but not always (Clerissi et al., 2020; King et al., 2019). This agrees with the 'Anna Karenina principle' for animal microbiomes, in which dysbiotic individuals vary more in microbial community composition than healthy individuals (Zaneveld et al., 2017). Finally, the potential role of bacteria from the *Rhodobacteraceae* family remains unclear. These bacteria were associated with juvenile oyster disease (Boettcher et al., 2005), but also with resistance to POMS (Clerissi et al., 2020).

During infection, the relative abundance of 4 OTUs belonging to the genera *Poseidonocella*, *Aureispira*, *Roseovarius* and *Olleya* increased under all conditions, suggesting that these bacterial genera are involved in the secondary phase of POMS, that is, colonization by opportunistic bacterial pathogens leading to the death of oysters (de Lorgeril et al., 2018). *Poseidonocella* and *Olleya* were overrepresented in *Ulva* spp. and in the oysters during acclimation and may have increased susceptibility to POMS. During infection we observed in oysters acclimated to green algae an increase in the relative abundance of *Vibrio*, a genus of bacteria previously associated with POMS (Clerissi et al., 2020; de Lorgeril et al., 2018; King et al., 2019; Petton, Bruto, et al., 2015). However, we did not observe this increase in oysters held with red *Solieria chordalis* and brown algae *Fucus vesiculosus*.

Although dysbiosis clearly appeared in oysters acclimated to *Ulva* spp., a causal relationship with viral proliferation in the host and increased mortality was not established. However, transcriptomic analysis revealed that oysters acclimated to these green algae overexpressed genes primarily related to the defence mechanism. Among these genes, we found laccase and tyrosinase, two enzymes of the phenoloxidase system involved in immune, antioxidant and detoxification responses in bivalves that potentially have a protective role against bacterial infection (Luna-Acosta et al., 2017; Quinn et al., 2020). Therefore, transcriptomic analysis supports dysbiosis as a major consequence for oysters held with green algae

More broadly, the transcriptomic analysis reveals that the number of differentially expressed genes (DEGs), including those related to the defence response, was highest in oysters acclimated to green algae *Ulva* spp., intermediate in oysters maintained with brown algae *F. vesiculosus* and lowest in oysters acclimated to red algae *S. chordalis*. Therefore, the number of DEGs seems to be associated with the risk of disease-induced mortality according to the different types of algae and is a good indicator of stress.

We further observe that red algae have a positive but weak effect on oyster survival. Considering that the statistical power is low, the experiment should be repeated with a higher number of replications

In conclusion, the present work shows that algae can alter the bacterial microbiota, immune status and disease outcome of oysters, thus highlighting the importance of considering macro and microbiotic interactions in disease, ecosystem and farm management. These considerations are particularly applicable to the development of integrated multi-trophic aquaculture, co-culture or any other practice that relies on increasing biological diversity to increase resilience of marine ecosystems to global change (see e.g. Falkenberg et al., 2021; Groner et al., 2018). We however suggest that future research should evaluate these interactions in natural field condition where oysters, OsHV-1 and seaweeds coexist.

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## CONFLICT OF INTEREST

We declare we have no conflict of interest

## AUTHORS' CONTRIBUTIONS

E.D., B.P. and F.P. designed the study and conducted the experiment; E.D, J.d.L., E.T. and Y.G. performed the bacterial microbiota and transcriptome analyses; E.D., J.d.L., E.T., Y.G. and F.P. analysed the data; E.D. and F.P. wrote the manuscript together. All authors contributed and approved the paper.

## DATA AVAILABILITY STATEMENT

Amplicon sequences for microbiota analysis and RNA-seq data have been made available through the SRA database (BioProject accession number PRJNA731543 with SRA accession SRP322069). Other data are submitted as electronic supplementary material and deposited at the SEANOE Digital Repository <https://doi.org/10.17882/85142> (Dugeny et al., 2021)



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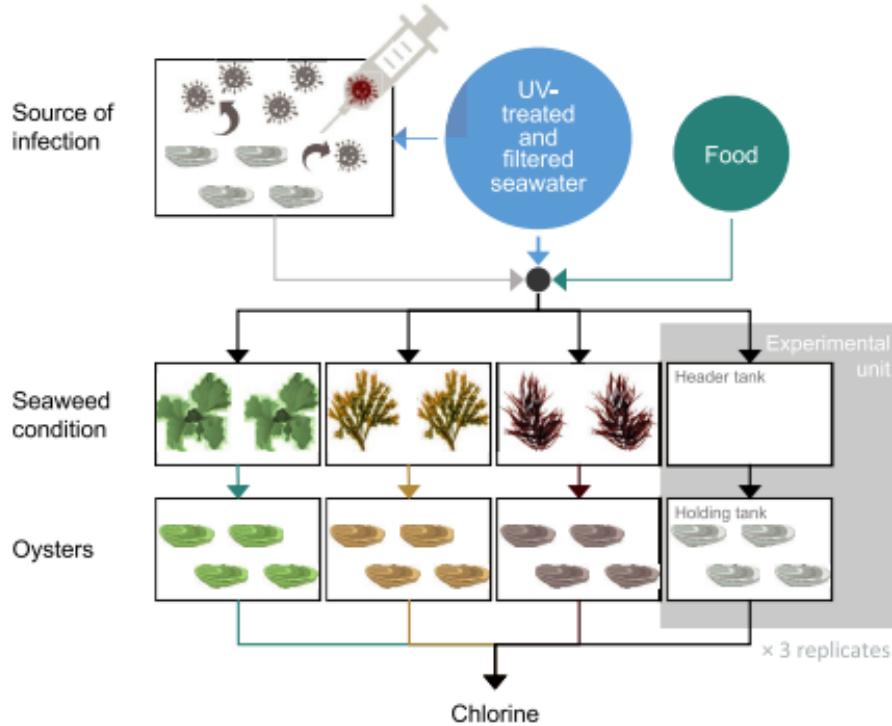
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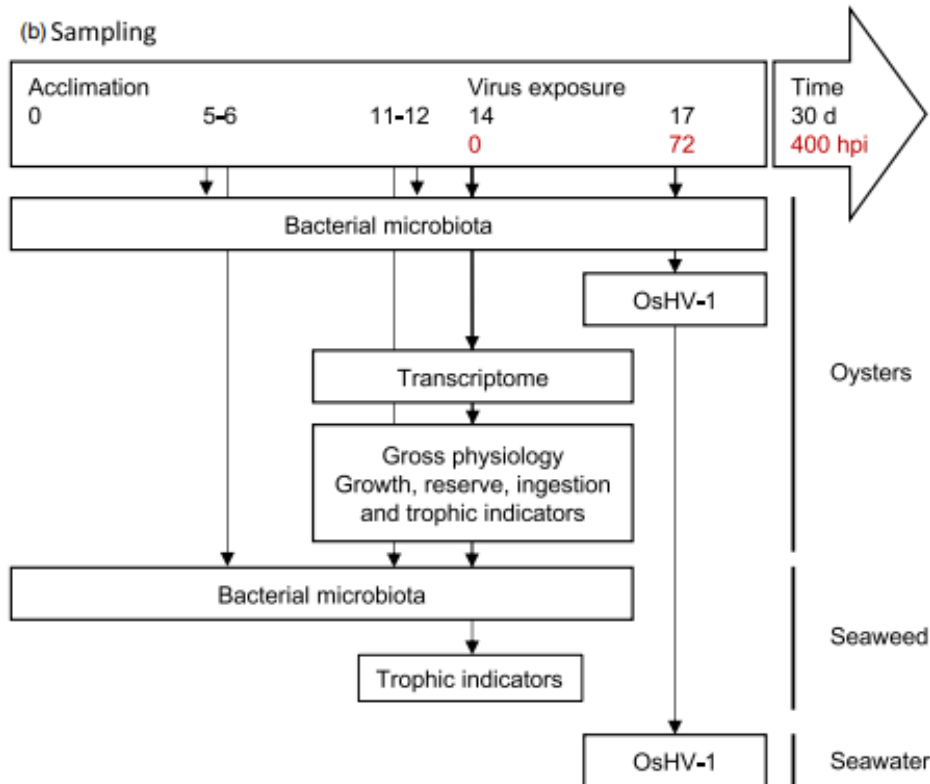
**FIGURE 1**

Experimental design (a) and sampling schedule (b). Only one replicate out of three is represented. Arrows indicate the direction of water flow. Each experimental unit consists of a header tank containing seaweed (or control) flowing to a holding tank containing oysters.

**(a) Experimental design**

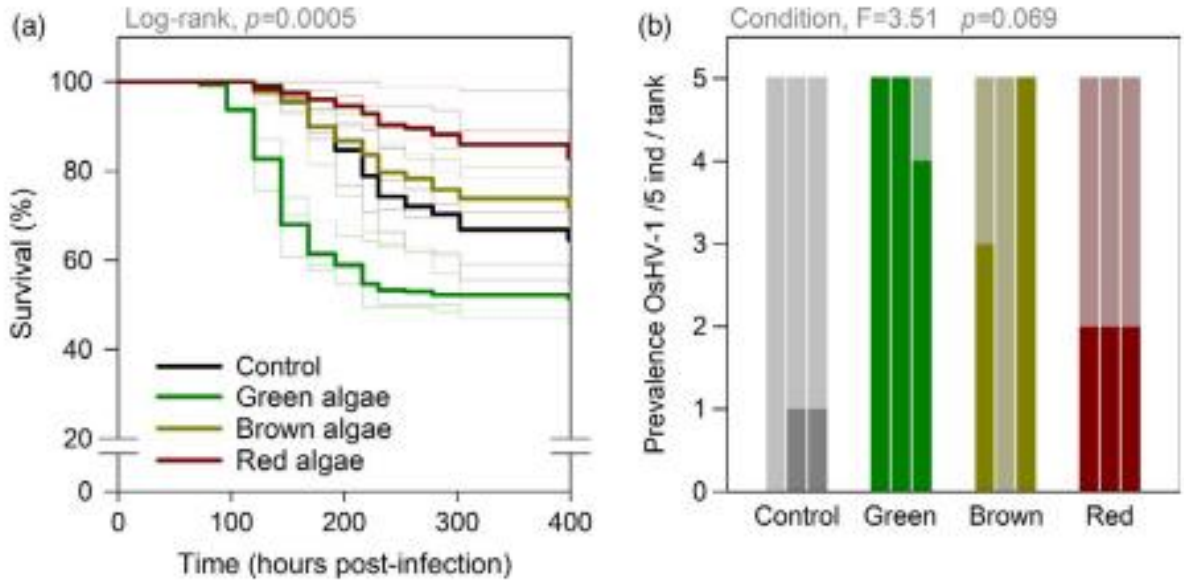


**(b) Sampling**



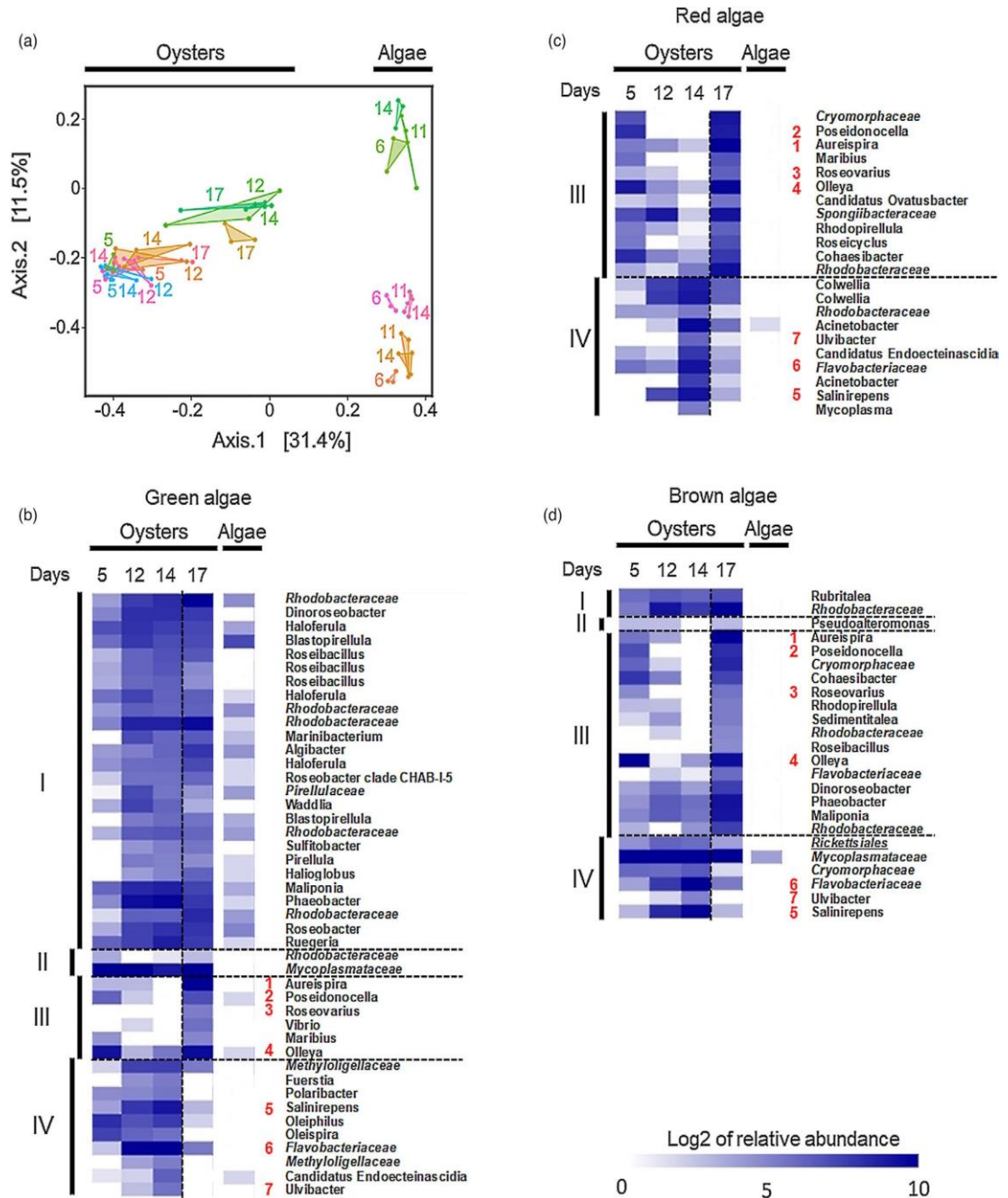
**FIGURE 2**

Survival of oysters exposed to OsHV-1 after acclimation with seaweed (a) and prevalence of OsHV-1 in oysters (b). The thin lines in graph A represent the survival curves for each individual tank while the thick line is the average by condition. The y-axis of graph B is the number of oysters positive to OsHV-1 out of 5 tested for each tank and condition 72 hpi. There are three bars per condition, each corresponding to one tank (replicate). The dark part of the bars represents positive individuals, while the light part represents individuals for whom the virus was not detected (negative). Star indicates statistically significant differences.



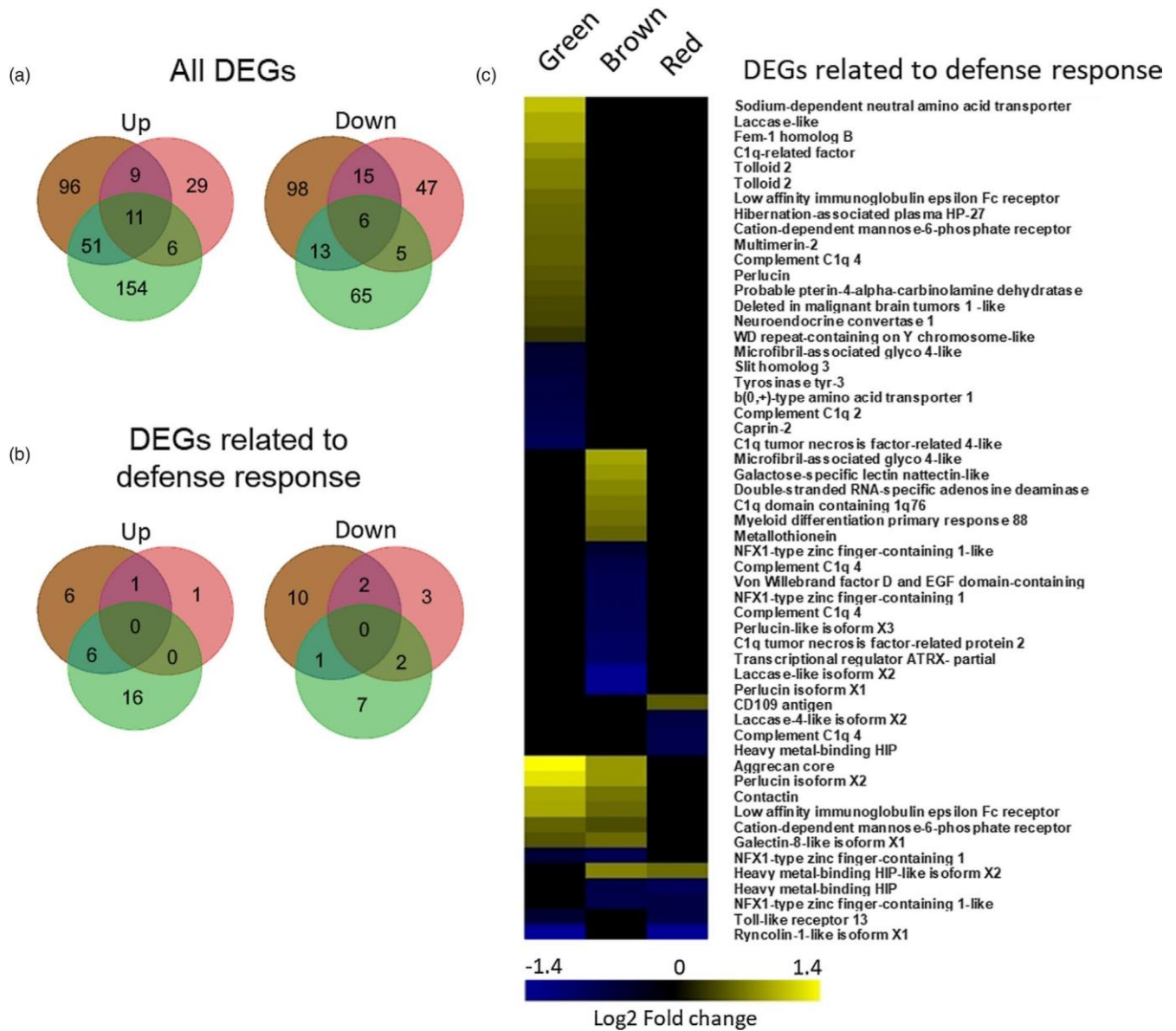
**FIGURE 3**

Bacterial microbiota in oysters during acclimation to seaweed and viral exposure. Multidimensional scaling (MDS) plot of the Bray–Curtis dissimilarity matrix of the microbiota (a). Each point of the triangles corresponds to one of the 3 replicates at one time. Dots represent distinct pools of five oysters. Colours correspond to algal species and numbers refer to days. Heatmaps of the bacterial taxa that were modified in oysters during acclimation to algae and viral exposure (b–d). Significant changes in taxa were evaluated by DEseq during acclimation (seaweed vs. control at 14 days) and viral exposure (17 vs. 14 days within algae). The numbers in red indicate taxa whose relative abundance was modified in all conditions during viral exposure. OTUs are classified according to the value of DEseq fold change (higher to lower) and clustered in four groups. Groups I and II include taxa whose relative abundances increased or decreased, respectively, during acclimation to algae, whereas groups III and IV include those that increased or decreased, respectively, during viral exposure. Analyses were performed at the genera level. The algae column corresponds to relative abundance of the bacterial taxa in algae at the beginning of the experiment (0 day) that were modified in oysters. The intensity level of the blue represents the relative abundance of bacterial taxa. At each time, the analysis was performed on three distinct pools of five oysters or three algae samples



**FIGURE 4**

Transcriptomic response of oysters. Venn diagrams of all differentially expressed genes (DEGs; a) and DEGs related to defence (b) relatives to control oysters. (c) Heatmaps of the enriched GO categories for DEGs related to defences (log<sub>2</sub> foldchange, p-adj < 0.05). The intensity of the enrichment is expressed as the ratio between the number of genes that were upregulated (yellow heat) or downregulated (blue heat) compared with the total number of genes in the category. If the intensity was equal to zero (black heat), then the enrichment was not significant





**TABLE 1**

Cox regression model for survival data of oysters acclimated to seaweed and exposed to OSHV-1. Control oysters with no algae were used as reference

Condition	<i>df</i>	Estimate	SE	$\chi^2$	<i>p</i>	Odds ratio
Brown	1	-0.203	0.360	0.318	0.573	0.816
Green	1	0.662	0.190	12.175	0.001	1.939
Red	1	-0.776	0.432	3.227	0.072	0.460

Abbreviations: SE, standard error; ddf, denominator degree of freedom.

**TABLE 2**

Levels of OsHV-1 DNA (cp/ mg) in oysters acclimated to seaweed and exposed to the virus 72 hpi. Data are means  $\pm$  SD (n = 3 tanks). Summary of ANOVA performed on log-transformed data. Means sharing a letter are not statistically different (Tukey-adjusted comparisons)

Condition	OsHV-1 DNA	ANOVA ( $F = 4.85, p = 0.033$ )				
		Estimate	SE	ddf	t value	p
Green	$3.3 \cdot 10^5 \pm 5.2 \cdot 10^5$	2.053 <sup>a</sup>	0.349	8	5.88	0.000
Brown	$7.7 \cdot 10^1 \pm 1.3 \cdot 10^2$	0.578 <sup>b</sup>	0.349	8	1.66	0.136
Red	$4.6 \cdot 10^4 \pm 8.0 \cdot 10^4$	0.906 <sup>b</sup>	0.349	8	2.60	0.032
Control	$2.7 \cdot 10^2 \pm 4.6 \cdot 10^2$	0.307 <sup>b</sup>	0.349	8	0.88	0.405

Abbreviations: SE, standard error; ddf, denominator degree of freedom.