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Vibrio splendidus infection induces dysbiosis in the blue mussel and favors pathobiontic bacteria

Yosra Ben Cheikh[a] and Marie Agnès Travers[b]

Authors affiliation

[a] University of Le Havre Normandy, Environmental Stress and Aquatic Biomonitoring, UMR-I 02 SEBIO, 25 rue Philippe Lebon, F-76063 Le Havre, France

[b] IHPE, Univ Montpellier, CNRS, IFREMER, Univ Perpignan Via Domitia, CC80, F-34095, Montpellier, France.

Corresponding Author

Yosra Ben Cheikh

E-mail: yosra.ben-cheikh@univ-lehavre.fr

Tel +33 (0)2 32 74 43 02

Abstract

Studies on marine epizootics are often based on the identification of a single pathogen. However, the one-pathogen-one-disease paradigm is not always sufficient to explain the disease, especially since the evidences on the role of microbiota in health and disease. *Vibrio splendidus* strains have been associated with *Mytilus edulis* mortality in France. To assess the role of mussel microbiota in the infectious process, we performed experiments combining the investigation of total microflora dynamics during a realistic experimental infection by *V. splendidus* and the monitoring of mussel survival and the dominance of potential opportunistic bacteria after antibiotic treatment. We found that Vibrio exposure affected the structure and predictive function of the mussel microbiota. Dysbiosis was accompanied with the appearance of a pathobiont dominated by Bacteroidetes and Fusobacteria phyla. The injection of a homogenate of infected organisms increased *Mytilus* mortality compared to the direct injection of Vibrio while the antibiotic pretreatment reduced the effect of pathogen exposure and mortalities. The decrease of opportunistic bacteria abundance in antibiotic pretreated animals confirmed their implication in pathogenesis. Our findings suggest that mussel disease results from a collaboration between external pathogens and pathobiont bacteria. Therefore, an insight into microbiota functions is needed to a better understanding of pathosystems.

Keywords:

Dysbiosis, mussels, pathogen, microbiota, Vibrio, antibiotics

Introduction

Marine epizootics are a major concern since they trigger widespread mass mortalities causing serious damages to the environment and the economy. These recurrent disease outbreaks are often difficult to understand and therefore to control notably because of their multifactorial character but also the struggles related to studying non-model organisms. Indeed, even if marine infectious diseases are usually associated to a causative agent, the implication of other factors including environmental conditions, host physiology or even the cooperation of microorganisms was demonstrated (Burge et al., 2014; Guo and Ford, 2016; King et al., 2019; Petton et al., 2021; Travers et al., 2015).

Among marine organisms, the blue mussel *Mytilus edulis* has been affected by massive mortalities since 2014 in French farms. The few investigations revealed the involvement of bacteria affiliated to *V. splendidus* and the detection of a disseminated neoplasia in mussels (Ben Cheikh et al., 2016; Benabdelmouna et al., 2018; Benabdelmouna and Ledu, 2016; Charles et al., 2020b). A more recent study suggested the combination of different factors such as environmental conditions, leading to a weakening of animals until death (Charles et al., 2020a). In our previous studies, we focused on the characterisation of bacteria isolates and the interaction of the virulent *V. splendidus* 10/068 1T1 with mussels at physiological and cellular levels. Using different experimental infection models, we demonstrated the capacity of the pathogen to impair host immune responses and to colonize mussel gills (Ben Cheikh et al., 2017, 2016). In this study, we propose to explore the interaction of this pathogen with host associated bacteria. It is hypothesised that the pathogen may disrupt the animal's homeostasis through the breakdown of the microbiota.

Nowadays, it is admitted that microbiota is a key element in maintaining organism homeostasis. Over the past years, research have been concentrated on human model demonstrating the crucial roles of commensal microflora in basic biological processes such as

digestion, metabolism and immunity. Besides the link with health, microbiota has been associated with major human diseases. Indeed, the alteration of microbiota composition induce human infectious and non-infectious diseases (for reviews see Wang et al., 2017; Zhang et al., 2015). More recently, the microflora of other vertebrate and invertebrate groups has been explored and similar observations have been made (Bourne et al., 2009; Cheng et al., 2015; Isaacson and Kim, 2012; Kong et al., 2014; Ogier et al., 2020; Thakuria et al., 2010). For example, microbiota influences behaviour of stinkbug nymphs and mice (Bravo et al., 2011; Hosokawa et al., 2008) and protect bumble bees against an intestinal parasite (Koch and Schmid-Hempel, 2011).

In marine bivalves, some commercial species were investigated for microbiota studies like the scallop *Pecten Maximus*, the clams *Ruditapes phlippinarum* and *Chamelea gallina* and in particular the oysters *Crassostrea* sp. and *Saccostrea glomerata* (Green and Barnes, 2010; Horodesky et al., 2020; Lasa et al., 2016; Leite et al., 2017; Milan et al., 2019). Microbial communities have been characterized in native population or under the influence of abiotic and biotic factors (Conceição et al., 2021; Lokmer et al., 2016; Wegner et al., 2013). In addition, many studies related oyster microbiota to disease events (for review see King et al., 2019). Recently, Lorgeril et al. (2018) demonstrated that Pacific Oyster Mortality syndrome (POMS) is caused by viral infection leading to a dysbiosis and subsequent bacteraemia by opportunistic bacteria. Furthermore, Clerissi et al. (2018) affirmed that microbiota composition and eveness might predict oyster mortalities. Unlike oysters, only few data are available for mussels. Some authors described bacteria associated to *Mytilus* (Musella et al., 2020; Utermann et al., 2018; Vezzulli et al., 2018) and others reported their shifts by environmental conditions, contamination and pathogens (Li et al., 2020, 2018, 2019a). However, the role of microbiota in mussel mortality outbreaks is still unknown.

The aim of this study is to explore the dynamics of microbial communities in blue mussels after exposure to the pathogen *V. splendidus* 10/068 1T1 and to investigate the link between the microbiota and mussel disease. Microbiota of whole animals was analysed during experimental infection by cohabitation assay. The involvement of autochthonous opportunistic microbes in disease process was explored by antibiotic treatment combined with experimental infection by injection to assess the mortality rate or a cohabitation experiment to quantify bacteria during infestation.

Materials and Methods

Mussel collection

Wild adult *M. edulis* were collected from the intertidal rocky shore of Yport (0°18'52"E:49°44'30"N, France) during September 2018 and June 2020. Animals were immediately transported to the laboratory and placed in a temperature-controlled (16°C) aerated water tank filled with seawater sampled from the site or in UV-treated and filtered seawater.

Experimental infection

The pathogenic GFP-tagged *V. splendidus* 10/068 1T1 strain was used in this study for experimental infection by injection and cohabitation assay as described previously (Ben Cheikh et al., 2017, 2016). Briefly, bacteria were grown overnight in Luria Bertani NaCl 20 g l^{-1} at 22°C with constant agitation (80 rpm) and resuspended in filtered sterile seawater (FSSW, OD_{600nm}= 1) after double washing (FSWW) /centrifugation (1200 g for 10 min).

Anesthetized mussels (2–3 h in magnesium chloride solution at 50 g l^{-1}, 4/5:1/5 v/v seawater/freshwater) were intramuscularly injected with a volume of 100 µl of bacterial suspension (2.10^8 CFU ml^-) or FSSW for negative controls and transferred to aerated tanks

filled with 2 l of UV-treated and filtered seawater supplemented with 50 ml of phytoplankton (*Isochrysis galbana*).

24h-post-infection, flesh of moribund mussels was homogenized in FSWW, coarsely filtered to eliminate tissue residues and injected intramuscularly to healthy animals (100 µl/animal). Mussels were maintained in the same conditions described above (3 tanks with 10 animals) during 10 days.

For cohabitation assay, moribund animals were sampled after 24h and placed for the cohabitation assay with apparently healthy mussels (3 moribund with 10 healthy/tank, 3 tanks) in tanks filled with the sampling site seawater (for microbiota analysis) or UV-treated and filtered seawater (for antibiotic experiment) and maintained under static conditions at 16°C with aeration during the experiment.

Mortality was monitored each day. Animals were considered moribund when after stimulation the valves closed little and dead when the valves did not. Newly dead mussels were removed from the tanks.

Antibiotic treatment

Mussels were injected intramuscularly with 100 µl of antibiotic solution mixture composed of 50 µl of penicillin 10000 U/ml - streptomycin 10 mg/ml (Gibco, Thermo Fisher Scientific, Massachusetts, USA) and 50 µl of metronidazole 10 mg/ml (Alfa Aesar™, Massachusetts, USA) three times a week, kept under stable conditions at 16°C with aeration and fed daily with phytoplankton. 3 days after the last antibiotic treatment, experimental infections were conducted by injection and cohabitation assays according to the protocol described above.

Sampling procedure and DNA extraction

Mussels were sampled after 2 days of cohabitation with moribund infected animals (Figure 1). Fifteen individuals were removed from their shells, flash frozen in liquid nitrogen and stored at -80°C. For microbiota analysis, control animals and seawater during the infection were also sampled. 5 l of seawater was filtered in series using porosity of 8 µm and 5 µm (Whatman™ Nucleopore™ Polycarbonate membrane filter, 47 mm diameter, UK). The 5 µm filtered seawater was then centrifuged 15 min at 11000 g. The membranes and bacterial pellet were stored at -80°C.

Frozen mussels were ground in liquid nitrogen. DNA was extracted from the animals and the bacterial pellet using NucleoSpin Tissue Kit (Macherey-Nagel, France)/Mag Bind® Universal Pathogen DNA Kit (Omega BIO TEK, Georgia, USA) and from the different filters using Quick DNA/RNA MagBead kit (Zymo Research, California, USA) according to manufacturer's protocol.

DNA concentration and quality were checked with the QuantiFluor® dsDNA System (Promega, France) and Nanodrop spectrophotometer (Thermo Scientific, Massachusetts, USA).

Bacteria quantification

Quantification of GFP-tagged *V. splendidus* 10/068 1T1, Fusobacteria strains and Bacteroidetes strains were performed using quantitative PCR (qPCR) analysis. Specific qPCR primers targeting GFP and 16S rRNA were designed using ProbeFinder software (https://lifescience.roche.com/ , Table 1). The GFP gene was chosen because it is specific to *V. splendidus* 10/068 1T1 and is stable over time (Ben Cheikh et al., 2016).

QPCR analysis was conducted on the Rotor-Gene Q 2-plex HRM (QIAGEN, Germany) using the QuantiTect® SYBR® Green Master Mix (2X, QIAGEN). Each reaction was performed in

duplicate with a final volume of 20 µl containing 100 ng genomic DNA and 0.5 µM of each primer. Reactions were initiated by initial denaturation for 15 min at 95 °C, followed by 45 cycles at 94 °C for 15 s, 59 °C for 30 s and 72 °C for 6 s. The melting curve was finally determined during a slow temperature elevation from 60 to 95 °C (1 °C s^{-1}). The run included blank controls (water). For the qPCR efficiencies of each primer pair used, specific standard curves were generated using eight serial dilutions of purified PCR products for 16S rRNA gene or genomic DNA for GFP gene (from 10^9 to 10^1 copies).

Microbiota analysis

Amplicon libraries construction, Illumina MiSeq sequencing and bioinformatics were performed by Biofidal (Vaulx-en-Velin, France). The 16S rRNA gene of the bacterial communities was amplified using the 5X HOT BIOAmp ® BlendMaster Mix (Biofidal) and the 341F: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATCTAATCC primers targeting the variable V3-V4 loops. After purification of PCR products by SPRIselect reagent kit (Beckman Coulter), a second amplification was performed using index adapter oligos (P5/P7). The purified PCR products were normalized to obtain an equimolar library and subsequently pooled. Negative controls were included during the library preparation. Amplicons were sequenced on the MiSeq platform with V3 reagents producing 2 X 300 bp paired-end reads. After demultiplexing, primers sequence adapters were trimmed from the reads using cutadapt software version 1.12 (Martin, 2011). Paired-end sequence reads were collapsed into so-called pseudoreads using sequence overlap with USEARCH version 9.2 (Edgar, 2010). After chimera and singletons removing, filtered pseudoreads were aligned to the RDP database version 11.5 (Cole et al., 2014) with Snap-aligner version 1.0 beta.23 (Zaharia et al., 2011) to identify the taxonomy. Finally, Usearch was used to create the OTU-table and to calculate alpha diversity. Principal coordinate analyses (PCoA) were performed via xlstat using the Bray–Curtis distance matrix.

Metagenomic functional prediction was performed with the Infer Functional Profile (beta) algorithm implemented in the Microbial Genomics module workflow (version 21.1) of the CLC Genomics Workbench (version 21.0.5). This tool is inspired by two published methods PICRUSt2 (Douglas et al., 2020) and Piphillin (Narayan et al., 2020). After the identification of EC abundance profile, a differential abundance analysis was performed using a pairwise Wald test to determine significance between control and infected mussels. Overabundant EC profiles with Bonferroni adjusted p value < 0.05, an absolute log2fold change =1.5, and a mean abundance ≥10 000 were retained.

All FastQ files were deposited in SRA-NCBI under the project accession number PRJNA735179.

Statistical analysis

SigmaPlot 12 (Systat Software Inc., Chicago, IL) was used for statistical analysis to compare alpha diversity metrics or bacteria quantification between conditions. Replicates were averaged and values were tested for normality (Shapiro-Wilk) then one-way ANOVA test was performed. When normality of residuals was rejected, the non-parametric Kruskal–Wallis test was performed. Pairwise comparisons between groups were assessed by post-hoc analyses (Holm-Sidak and Tukey test). Kaplan Meier analyses and survival curves were computed with R scripts (survminer). Statistical significance was accepted for $p < 0.05$ or $p < 0.001$.

Results

Selection of infected mussels and microbiota analysis

Wild mussels were exposed to the virulent GFP-tagged *V. splendidus* 10/068 1T1 by cohabitation experiment and sampled 48h post-infection. At this time, no mortality was observed. The success of infection was assessed by quantification of GFP-tagged *V.*

splendidus DNA (qPCR) in animal tissues and positive individuals were selected for microbiota analysis (Figure 1).

Microbial communities were sequenced using the 16S rRNA gene from 15 mussels from each condition (control and infected) and from seawater sampled 48h post-infection (2 filters and bacterial pellet). The sequencing run generated a total of 3 830 359 raw reads with a minimum of 78 552 reads to a maximum of 154 343 reads. After Chimera filtering, unique pseudoreads represented in average 46 705 and 2078 OTUs.

Variability of microbiota across different mussels and seawater

Overall, the bacterial community composition at the phylum level was similar between replicates and different between conditions (control, infected and seawater). Control mussels showed 9 diverse phyla with an abundance > 1% and the predominance of Proteobacteria, (51.5% ± 3.4%) and Cyanobacteria (12.5% ± 1.4%). Rare phyla presented a relative abundance between 1.3% ± 0.2% and 7.1% ± 1.2% (Firmicutes, Planctomycetes, Bacteroidetes Actinobacteria, Verrucomicrobia, Tenericutes and Chloroflexi, Table 2, Figure 2 A). Exposure of mussels to *V. splendidus* 10/068 1T1 induced a change in microbiota composition (Table 2, Figure 2 B) with only 4 phyla showing an abundance > 1%. Comparison of the infected group with the control group revealed an increase in the relative abundance of Proteobacteria (59.5% ± 2.8%, not significant data), Bacteroidetes and Fusobacteria (respectively 20.6 % ± 1.9% and 14.9% ± 2%, $p<0.001$) and a significant and drastic decrease in relative abundance in the other phyla described in the control group (<1%, $p<0.001$). The bacterial phyla detected in the seawater during infection were more similar to the infected mussels, with 3 phyla showing an abundance > 1% (Table 2, Figure 2 C). Compared to the infected animals, the seawater was characterized by a higher proportion of Proteobacteria (78.2% ± 3.5%, $p<0.05$) and Firmicutes (2.9% ±0.3) and a lower proportion of Bacteroidetes (18.3% ± 3.4%) and Fusobacteria (<1%, $p<0.01$).

Comparative analysis of the most abundant families (top 30) and genera (top 20) in the samples revealed the diversity of bacteria in control mussels, infected mussels and seawater (Supplementary Table S1, Figure 2). The control animals were mainly dominated by Rickettsiaceae family (16.9% ± 4.4%)/Candidatus Megaira genus, Rhodobacteraceae family (8.3% ± 2.1%)/ Labrenzia genus, Planctomycetaceae family (5.8% ± 4.4%)/ Blastopirellula, Pirellula, Planctomyces genus, Flavobacteriaceae family (3.8% ± 1%)/ Lutimonas genus and unclassified Cyanobacteria (9.2% ± 2.4%). During the infection process, the predominant bacterial communities were represented by Moraxellaceae family (6.4% ± 0.8%)/ Acinetobacter genus, Shewanellaceae (6.2% ± 0.8%)/ Shewanella genus, Marinilabiliaceae family (10.3% ± 2.1 %)/ Marinifilum genus, Fusobacteriaceae family (14.9% ± 2%)/Psychrilyobacter genus and unclassified Epsilonproteobacteria (25.4% 2.1%). No change was observed for Vibrios. Seawater samples were enriched by Flavobacteriaceae family (11.7% ± 0.9%)/ Flavobacterium and Chryseobacterium genus, Caulobacteraceae family (8.2% ± 4.5%)/ Brevundimonas genus, Comamonadaceae (7.7% ± 3%)/ Malikia genus as well as Pseudomonadaceae family (7% ± 1.4%)/ Pseudomonas genus. Furthermore, communities belonging to Moraxellaceae family (22.2 % ± 4.6%)/Acinetobacter genus were more abundant in the seawater than in the infected animals.

Microbial diversity was also analysed in order to describe diversity within a sample (Alpha-diversity) or between samples (Beta-diversity). Alpha diversity metrics confirmed the difference of richness and diversity between samples. Compared to control mussels, the Chao 1 index was significantly lower in the infected group and in seawater ($p<0.001$) while no significant difference was observed between infected mussels and seawater (Figure 3 A). Similarly, a significant decrease in the Shannon index was observed in mussels after infection with *V. splendidus* (Figure 3 B, $p<0.05$). Whereas, seawater samples showed a slightly higher Shannon index than the infected group and lower than the control group (not significant).

Principal coordinate analysis (PCoA) using Bray-Curtis distance matrices indicated dissimilarity between control, infected mussels and seawater. The replicates were grouped into separate clusters showing the shaping of the bacterial community composition of the mussels by *V. splendidus* infection and the divergence of the seawater samples (Figure 3C).

Variability of functional prediction in controls and infected mussels

To assess the potential functionality of the pathobiont in *V. splendidus* infection, the corresponding metagenomes were inferred from the taxonomic profiles. A differential abundance analysis of identified enzymes in control and infected mussels was carried out resulting in the gene abundance of 29 enzymes in controls and 32 in infected animals (Bonferroni adjusted $p < 0.05$, Figure 4). Overall, the enzymes identified in the infected group were originally characterized from pathogenic bacteria (Supplementary Table S2).

Functional profiles differed from control to infected mussels and clustered in 23 pathways common or specific to each group. Control animals were characterized by enrichment in pathways related to photosynthetic bacteria (chlorophyll metabolism, carbon fixation or carotenoid biosynthesis) and more classical pathways involved in organic solute biosynthesis, glycolysis, nucleotide degradation and nitrogen or sulfur metabolism. In contrast, infected mussels clustered an abundant pathway represented by vitamin metabolism including enzymes involved in menaquinone and thiamine synthesis and other pathways involved in compound conjugation, the TCA cycle, ion transport, amine and polyamine biosynthesis and the metabolism of DNA, glyoxylate and dicarboxylate, bile acids and propanoate.

Both groups of animals shared some pathways but with different enzymes, such as anaerobic respiration, degradation of aromatic compound or cell wall biosynthesis. Among the represented pathways, amino acid metabolism was also overrepresented and included enzymes implicated in the synthesis or degradation of several amino acids (arginine, proline,

glycine, serine, threonine, valine, leucine isoleucine, lysine, arginine for controls and aspartate, glycine, isoleucine, lysine, selenoamino acid for infected mussels). Protein metabolism was more represented in infected animals and was related to protein glycosylation (glutathione synthesis in controls). In contrast, carbohydrate metabolism was more abundant in controls and was related to sucrose and aminosugar metabolism (glucose in infected animals).

Mussel mortalities among antibiotic treatment and *V. splendidus* infection

In order to investigate whether opportunistic pathogens contribute to the infection process, a group of mussels was treated with a cocktail of antibiotics (penicillin-streptomycin-metronidazole) targeting anaerobic bacteria in particular Fusobacteria and Bacteroidetes (Cheung and Bellas, 2007; Löfmark et al., 2010) then experimental infection with *V. splendidus* 10/068 1T1 was performed by injection assay. No mortality was observed during antibiotic treatment. Exposure to the pathogen *V. splendidus* at the lethal dose induced 23.3% ± 3.3% and 33.3% ± 6.7% mortality in the pretreated mussels after 2 and 10 days respectively and 73% ± 3.3% and 80% ± 10% mortality in the untreated group. Injection of a homogenate of infected animals induced 100% mortality after 24h (Figure 5).

Effect of *V. splendidus* infection and antibiotic treatment on bacteria abundance in mussels

To establish a link between opportunistic bacteria and mortality rates, mussels pretreated or not with antibiotics were exposed to GFP-*V. splendidus* 10/068 1T1 in a cohabitation assay for 48h. Six strains belonging to the phyla Fusobacteria and Bacteroidetes as well as the pathogen *V. splendidus* 10/068 1T1 were quantified in the different groups exposed to *Vibrio* and in the control animals (Figure 6).

Overall, the selected bacterial strains were weakly detected or not detected in the control mussels excepting for *Flavobacterium* sp. ($3.07x10^3 \pm 1.18x10^3$ copies). Infection with *V. splendidus* induced a significant increase of all quantified bacteria, in particular the Fusobacteria strains *Psychrilyobacter* sp. and *Ilyobacter* sp. and the Bacteroidetes strains *Bacteroides* sp. and *Marinifilum* sp. (respectively 833-, 612- ,1579- and 125- fold higher than the control, $p<0.001$). The pathogen *V. splendidus* was also abundant in the animal tissues ($7.93x10^4 \pm 1.89x10^4$ copies). Compared to untreated group, the injection of antibiotics affected the abundance of bacteria in mussels during *Vibrio* infection, significantly decreasing the proliferation of five out of seven bacterial species. *Psychrilyobacter*, *Ilyobacter* and *Marinifilum* sp. were respectively 38-, 30- and 8-fold lower in the treated group compared to untreated animals but still higher than controls ($p<0.001$). Likewise, *Propionigenium* and *Bacteroides* levels were reduced and similar to those of the control mussels. On the other hand, antibiotic treatment had no significant effect on *Flavobacterium* sp. but significantly increased the abundance of *V. splendidus* (1000-fold higher, $p<0.001$).

Discussion

For a long time, infectious diseases have been linked to a single external pathogen that infests its host causing damage. However, this view has been controversial since recent findings have highlighted the polymicrobial character of several diseases, in some cases implicating the microbiota (Vayssier-Taussat et al., 2015, 2014). In our study, we explored the interactions of the blue mussel pathogen *V. splendidus* 10/068 1T1 with the host microbiota and demonstrated the contribution of the pathobiont in mussel disease.

Vibrio splendidus infection induces dysbiosis in *Mytilus edulis*

Analysis of the total microbiota revealed that healthy mussels are dominated by Proteobacteria (51.5 %) followed by Cyanobacteria (12.5%). Firmicutes, Planctomycetes,

Bacteroidetes Actinobacteria, Verrucomicrobia, Tenericutes and Chloroflexi appeared as subdominant phyla with a relative abundance ranging from 7.1% to 1.3%. The presence of these microbial phyla is common in marine bivalves such as oysters (King et al., 2012; Lokmer et al., 2016) and mussels (Musella et al., 2020; Pierce and Ward, 2019; Rubiolo et al., 2018). However, the prevalence and pattern of dominant bacteria are difficult to compare across studies, especially at lower taxonomic levels, as the structure and the composition of the microbiota is particularly influenced by host factors (genetics, life stages, health status) and harvesting environments (ecology and geography, season) (Paillard et al., 2022). Indeed, previous research has revealed the change in microbiota composition in mussels by different factors such as diet, temperature (Li et al., 2019b, 2018), season (Pierce and Ward, 2019) and depuration (Rubiolo et al., 2018; Vezzulli et al., 2018). Moreover, Musella et al. (2020) showed the variability of microbial communities in *Mytilus galloprovincialis* between the digestive gland, foot, gill, stomach and hemolymph. Our study reports for the first time the investigation of whole-body microbiota in *Mytilus edulis* and comparison with other studies is thus difficult.

Besides environmental factors, the microbiota can be modulated by pathogens leading to its collapse and the emergence of pathobiont as described in many infectious diseases affecting humans, animals and plants (Bass et al., 2019; Surana, 2019; Wang et al., 2017). Microflora imbalance or dysbiosis is often related to a reduction in microbial density, membership composition, and overall changes in community structure and function. Nevertheless, in some cases we can observe no change or an increase of diversity in diseased organisms due to pathobiontic communities (Mitra et al., 2015). For example, in the coral *Cladocora caespitosa*, microbial communities were similar in healthy and necrosed samples (Rubio-Portillo et al., 2018).

In our study, the exposure of mussels to the pathogen *V. splendidus* 10/068 1T1 induced a shift in bacterial communities resulting in a decrease in diversity and richness. Indeed, the proportion of the most representative phyla severely declined in infected animals in favour of bacteria belonging to Bacteroidetes phylum i.e., Mangroviflexus, Bacteroides and Marinifilum, or to Fusobacteria phylum i.e., Psychrilyobacter. While a slight non-significant increase was observed in Proteobacteria, the composition of bacteria changed with a proliferation of unclassified Epsilonproteobacteria and Acinetobacter/Shewanella/Arcobacter genera replacing Candidatus Megaira genera and unclassified Gammaproteobacteria and Alphaproteobacteria strains. Interestingly, the proportion of Vibrio appeared stable in the infected group, but a dominance of some virulent species i.e., *V. tapetis, V. kanaloe*, was recorded (data not shown). The seawater sampled during the infection was differentiated from the mussels with a dominance of Proteobacteria bacteria such as Acinetobacter and Brevundimonas followed by Bacteroidetes bacteria such as Flavobacterium.

A similar destabilisation of the microbiota has been described in some marine bivalves after infection with pathogens. For example, *V. orientalis/tubiashii* species increased the variation in hemolymphatic community composition in dead Pacific oysters as well as anaerobic bacteria, including members of Clostridia. In accordance with our findings, moribund oysters microbiome were dominated by the genus Arcobacter and, not as expected, by Vibrio and harboured more strains of other genera with potentially pathogenic species described such as Shewanella (Lokmer and Wegner, 2015). Likewise, exposure of *M. coruscus* to *V. cyclitrophicus* reduced the diversity of hemolymphatic microbiota promoting the proliferation of opportunistic pathogens e.g., Arcobacter and Francisella (Li et al., 2019a). More recently, deciphering of Pacific Oyster Mortality Syndrome revealed the proliferation of Proteobacteria such as Arcobacter and Vibrio but also strains belonging to Fusobacteria phylum after viral infection (Lorgeril et al., 2018; Lucasson et al., 2020).

In addition to the phenotypic imbalance, metagenomic functional prediction analysis revealed a change in microbial pathways after *V. splendidus* infection with a predominance of vitamin, amino acid and protein metabolism. Indeed, infected mussels showed a gene abundance of enzymes implicated in the biosynthesis of menaquinone (vitamin K2, MK) via the futalosine pathway. MK is a small redox molecule that is essential for energy generation in many bacteria. In the human pathogen *Mycobacterium tuberculosis*, MK also plays a role in triggering persistence through its ability to signal the redox state (Bashiri et al., 2020; Honaker et al., 2010). MK can be synthesized by anaerobic members of human intestinal microbiota including Bacteroides which is among the overrepresented groups in infected mussels (Ramotar et al., 1984). The abundance of the amino acid metabolism pathway in Vibrio exposed animals is not surprising given its importance for bacterial growth, especially during infection, and may suggest the unavailability of these amino acids in the environment of proliferating bacteria. For example, de novo synthesis of histidine is crucial for the growth and the pathogenesis of *M. tuberculosis* (Dwivedy et al., 2021) and bacterial synthesis of aspartate is absolutely essential for staphylococcal survival in bone (Potter et al., 2020). Interestingly, the enzymes identified in the infected group, implicated in the biosynthesis or degradation of aspartate, play a role in the virulence of pathogens. Indeed, aspartate racemase acts as a positive regulator of biofilm formation and motility in Salmonella while aspartate ammonia-lyase increases the acid survival of enteric bacterium *Yersinia pseudotuberculosis* by producing ammonia (Hu et al., 2010; Iyer, 2018).

The third pathway overrepresented in infected mussels concerns protein metabolism, in particular the *N*-linked protein glycosylation (*pgl*) system identified in *Campylobacter jejuni*, which has been related to stress resistance, biofilm formation, motility and chemotaxis (Cain et al., 2020). Moreover, other enzymes in underrepresented pathways have been correlated with pathogenic bacteria like aryl-sulfate sulfotransferase which is frequently found in

pathogens and is upregulated during infection (Malojčić et al., 2014), carboxynorspermidine decarboxylase indispensable for biofilm formation in *V. cholera* (Lee et al., 2009), the nickel ABC transporter involved in nickel uptake required for pathogen colonization including *Staphylococcus aureus* and the human gastric pathogen *Helicobacter pylori* (Tanaka et al., 2018) and the enzymes UDP-2-acetamido-2-deoxy-ribo-hexuluronate aminotransferase, UDP-N-acetylglucosamine 4,6-dehydratase involved in the synthesis of functional flagella and lipopolysaccharides in *H. pylori* (Ishiyama et al., 2006). Interestingly, biofilm formation and flagellar systems have been identified as responsible for the virulence and are important for the infection and colonisation of several Vibrio species such as *V. coralliilyticus* and *V. splendidus* (Liang et al., 2022; Ushijima and Häse, 2018).

Opportunistic bacteria contribute to *Mytilus edulis* infection

If the relevance of pathobiont has been demonstrated in marine organisms during pathogen infection, the role of these community members in disease establishment still unclear (Paillard et al., 2022). For mussels, besides the massive proliferation of Fusobacteria and Bacteroidetes during Vibrio infection, injection of a homogenate of infected animals increased mortality rates (100% of mortality after 24h) compared to the injection of *V. splendidus*, suggesting the implication of other pathogens in the disease. Some species belonging to the Fusobacteria and Bacteroidetes are known to be opportunistic bacteria in humans i.e., the oncogenic *Fusobacterium nucleatum* and *Bacteroides fragilis,* but these phyla remain poorly described in marine organisms (Brennan and Garrett, 2019; Wexler, 2007). Fusobacteria represent a major bacterial group in the core gut microbiome of some marine and freshwater fish (Roeselers et al., 2011; Tarnecki et al., 2017) and genera such as Psychrilyobacter have been described during oyster disease (Lorgeril et al., 2018). Members of the phylum Bacteroidetes such as Marinifilum have been described in shrimps and lobsters (Holt et al., 2020; Tepaamorndech et al., 2020).

Antibiotic pretreatment confirmed the contribution of pathobiontic communities to pathogenesis. Indeed, animals injected with a cocktail of antibiotics, targeting anaerobic bacteria, contained lower levels of Fusobacteria and Bacteroidetes strains and better survived *V. splendidus* infection. In accordance with our findings, a few studies have reported reduced mortality in antibiotic-treated oysters exposed to OsHV-1 demonstrating the contribution of opportunistic Vibrio to POMS. Moreover, authors confirmed that bacterial colonization was a second event necessary to complete the infectious process leading to death in oysters (Lorgeril et al., 2018; Petton et al., 2015). A similar scenario implicating primary infection by the pathogen *V. splendidus* complemented by subsequent colonization by opportunistic anaerobic bacteria seems plausible for mussel disease. However, to fully understand this process, the nature of the interactions between the external pathogen, the pathobiont and the host must be elucidated. In our previous study, we demonstrated the ability of *V. splendidus* to impair the immune defense of mussels, which may be advantageous to pathobiontic bacteria (Ben Cheikh et al., 2017, 2016). Indeed, by definition, opportunistic bacteria are dormant microorganisms that become pathogenic following a perturbation in their host environment, including immune suppression (Brown et al., 2012). The decline in mortality in antibiotic-treated mussels despite the increase in abundance of *V. splendidus* suggests that opportunists are exploiting the pathway paved by Vibrio at the detriment of Vibrio. Furthermore, we might think that pathobiont enhances the virulence of Vibrio sp. especially since the experimental infection by bathing did not cause mortality unlike the cohabitation and injection assays (Ben Cheikh et al., 2017). Recent studies have demonstrated that some microbiota can lead to the expansion or enhancement in virulence of pathogenic populations (Cameron and Sperandio, 2015).

Conclusion

In conclusion, the present work has described the interactions between the pathogen *V. splendidus* 10/068 1T1 and the mussel microbiota underlying the role of the pathobiont in *Mytilus edulis* disease. We demonstrated the transition of the total mussel microbiota from a balanced to a dysbiotic status during pathogenesis. In addition to the phenotypic imbalance, Vibrio infection induced a change in the predicted function of the microbiota by enhancing gene abundance of enzymes implicated in pathogen virulence mechanisms. The involvement of opportunistic bacteria in the pathosystem was demonstrated using antibiotic treatment which enabled colonization of certain pathobiont strains and reduced mortality rates after infection. These results highlight the polymicrobial character of mussel disease and provide a new insight into marine epizooties. However, major efforts are needed to decipher the biological roles of these poorly studied pathobiontic communities and their relationship with the host.

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Conflict of interest

The authors declare no competing interests.

Appendix A.

Supplementary table 1

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Figure legends

Figure 1. Experimental design for microbiota analysis. Mussels were infected with GFP-tagged *V. splendidus* 10/068 1T1 by a cohabitation assay and mortality was monitored daily (data are mean ± SEM of cumulative mortalities in triplicate tanks, 10 mussels/tank). 48h post cohabitation, mussels were sampled, flash frozen and after DNA extraction, GFP-tagged *V. splendidus* 10/068 1T1 was quantified by qPCR. GFP-*V. splendidus* positive mussels were selected for microbiota analysis using 16S rRNA gene-metabarcoding.

Figure 2. The relative abundance of bacterial communities in control mussels, infected mussels and seawater. A-C) bacterial phyla, D-F) heatmaps revealing the top 30 bacterial families, the left color bar indicates the bacterial phyla and G-I) the top 20 bacterial genera. Replicates are labelled with numbers 1 to 15 for animals and 1 to 3 for seawater.

Figure 3. Alpha and beta diversity in mussels and seawater during infection. A) and B) alpha diversity expressed as Chao1 and Shannon index. Data are mean ± SEM (n=15 for mussels, n=3 for seawater). Different letters indicate significant differences ($p<0.05$, $p<0.001$, ANOVA and Kruskal-Wallis tests), C) beta diversity calculated using Principal Component Analysis (PCoA) applied to the Bray-Curtis distance matrix.

Figure 4. Differential abundance of inferred enzymes in control and infected mussels. Wald test, Bonferroni adjusted p value < 0.05, absolute log2 fold change =1.5, mean abundance ≥10000.

Figure 5. Survival curves of *V. splendidus*-infected mussels without or with antibiotic treatment. Mussels were injected intramuscularly with the pathogen *V. splendidus* 10/068 1T1 or with a homogenate of infected animals or with antibiotics (3 doses /week) then *V. splendidus* 10/068 1T1 at lethal dose (OD_{600}=1). The curves represent the average cumulative mortality in triplicate tanks (10 mussels/tank). Confidence intervals are also represented.

Figure 6. Effect of *V. splendidus* infection and antibiotic treatment on bacteria abundance in mussels. The pathogen GFP-tagged *V. splendidus* 10/068 1T1 and six core bacteria were quantified in mussels (by qPCR) before (Ctrl) and 2 days post-cohabitation experiment (Inf). Inf + antib indicates mussels pre-injected with antibiotics (3 doses /week) and then exposed to *V. splendidus* by a cohabitation assay. Data are means ± SEM (n=15). Different letters indicate significant differences ($p<0.001$, ANOVA).

Table 1. Primers used for bacteria quantification

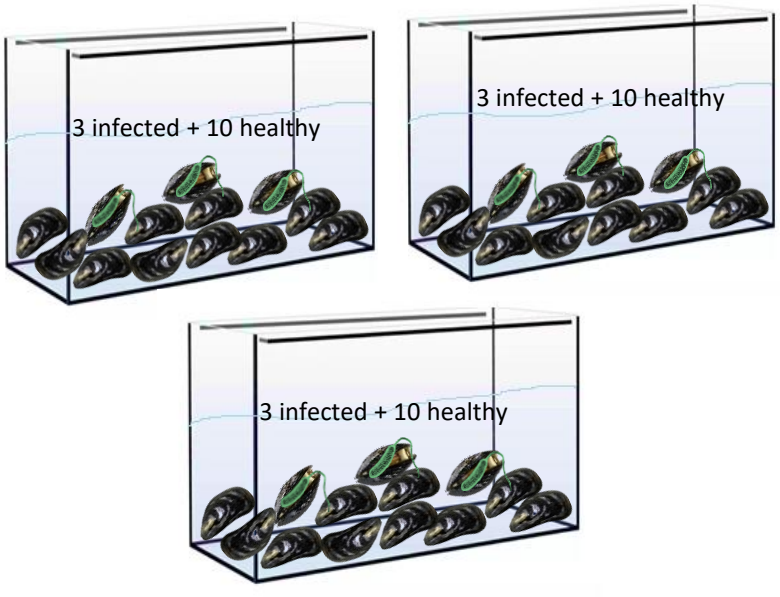
Strain	Target gene	Accession number	Fw 5'-3'	Rev 5'-3'	Amplicon size
V. splendidus 10/068 1T1	GFP	KX506099.1	TCAGTGGAGAGGGTGAAGGT	CCATGCCCGAAGGTTATGTA	194 pb
Psychrilyobacter atlanticus	16S rRNA	LS999394.1	GACGCTAAAGCTCGAAAGCG	GTTAGCTTCGGCACAGAGGT	112 pb
Propionigenium maris	16S rRNA	MK999892.1	TATCAGAGAGGTGGGCGGAA	AGTGAGCTGGCTTCCCAATC	84 pb
Ilyobacter psychrophilus	16S rRNA	AJ877255.1	TTTGCCCTTCAGACTGGGAC	CGCAAAGTTCTCCTTCAGCG	111 pb
Bacteroides graminisolvens	16S rRNA	MK039103.1	TGGTGTAGCGGTGAAATGCT	CGAGCCTCAGTGTCAGTTGT	85 pb
Marinifilum flexuosum	16S rRNA	MK674861.1	AGTTGAGGTGGGCGGAATAC	TTAGTGAGCTGCCTTCGCAA	83 pb
Flavobacterium anatoliense	16S rRNA	NR_109424.1	TCGATGATACGCGAGGAACC	GACAACCATGCAGCACCTTG	100 pb

Table 2. Variability of bacterial phyla across different mussels and seawater

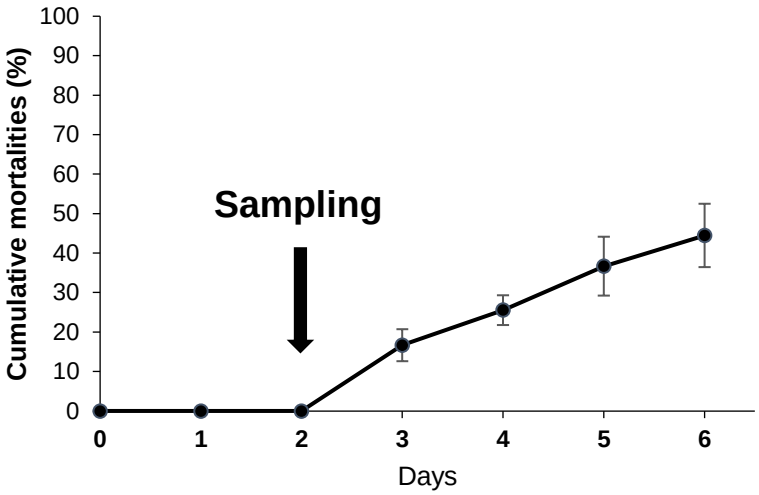
Phylum	Control	Infected	Seawater
Proteobacteria	51.5 ± 3.4 ^{a*}	59.5 ± 2.8 ^{ab}	78.2 ± 3.5 ^{b*}
Cyanobacteria	12.5 ± 1.4 ^{a***}	1.3 ± 0.2 ^{b***}	< 1 ^{bc**}
Firmicutes	7.1 ± 1.2 ^{a***}	< 1 ^{b***}	2.9 ± 0.3 ^{bc***}
Planctomycetes	6.2 ± 0.7 ^{a***}	< 1 ^{b***}	< 1 ^{bc***}
Bacteroidetes	5.2 ± 1.1 ^{a***}	20.6 ± 1.9 ^{b***}	18.3 ± 3.4 ^{bc*}
Actinobacteria	4.6 ± 0.5 ^{a***}	< 1 ^{b***}	< 1 ^{bc*}
Verrucomicrobia	3.5 ± 0.4 ^{a***}	< 1 ^{b***}	< 1 ^{bc***}
Tenericutes	2.5 ± 0.8	< 1	< 1
Chloroflexi	1.3 ± 0.2 ^{a***}	< 1 ^{b***}	< 1 ^{bc**}
Fusobacteria	< 1 ^{a***}	14.9 ± 2 ^{b***}	< 1 ^{ac**}

Data are percentage of relative abundance ± SEM, different letters indicate significant difference between samples: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

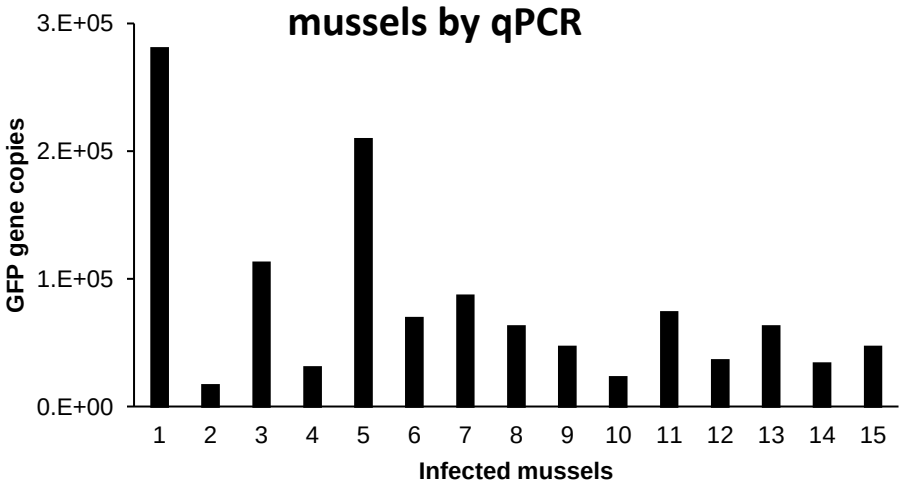
1. Cohabitation experiment



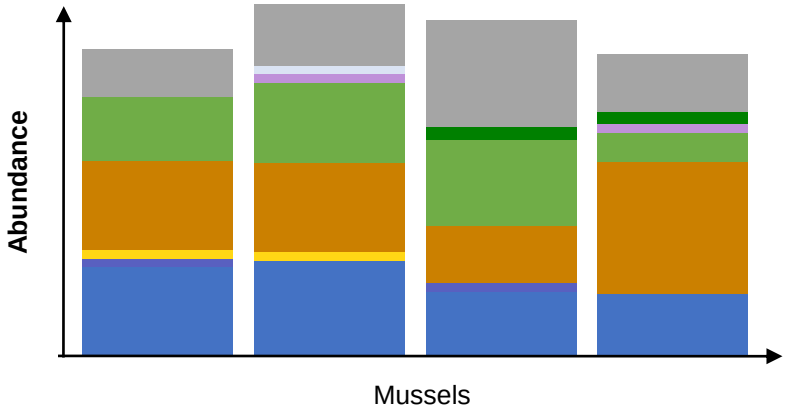
2. Monitoring of mortalities post cohabitation and sampling

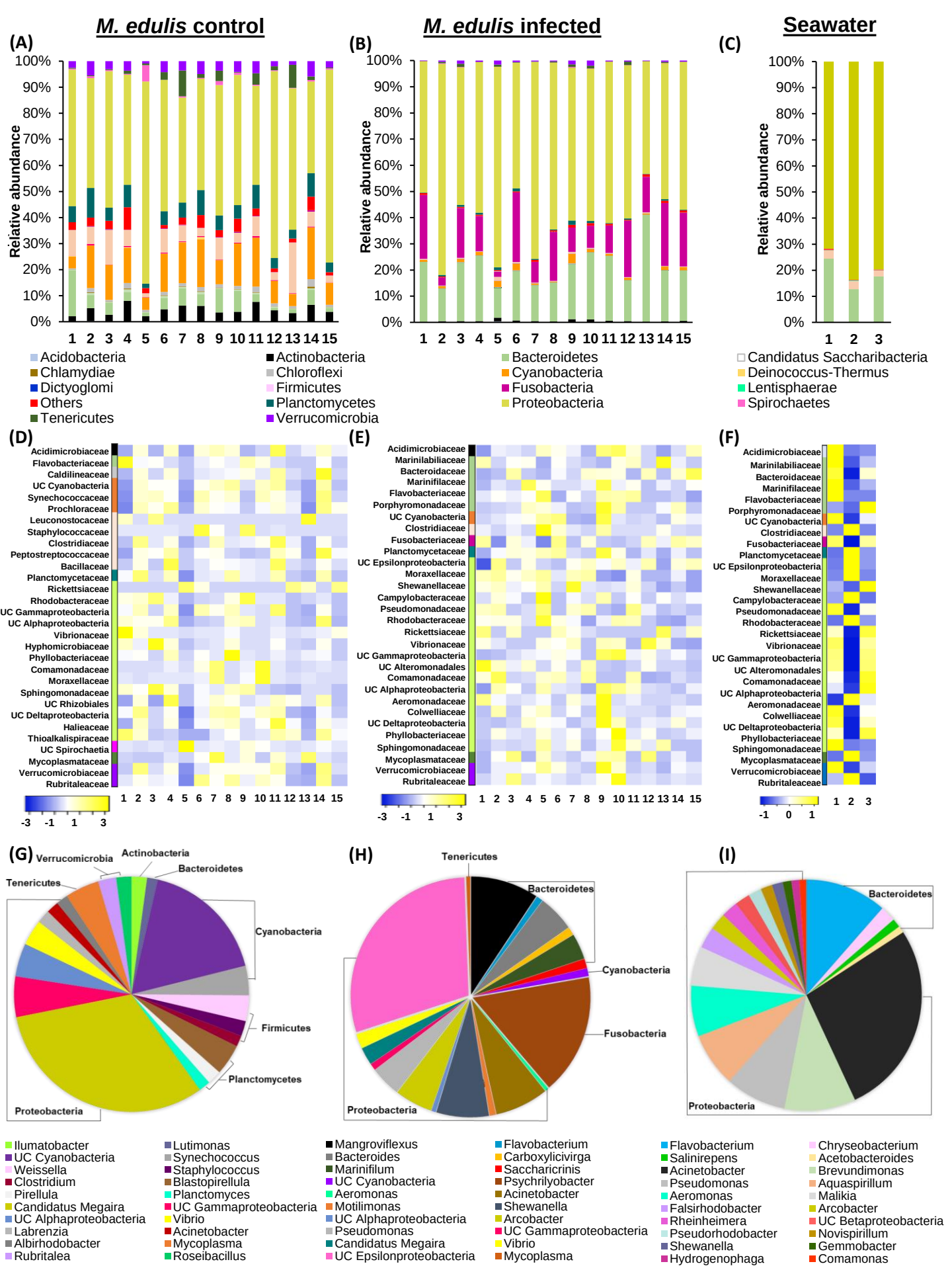


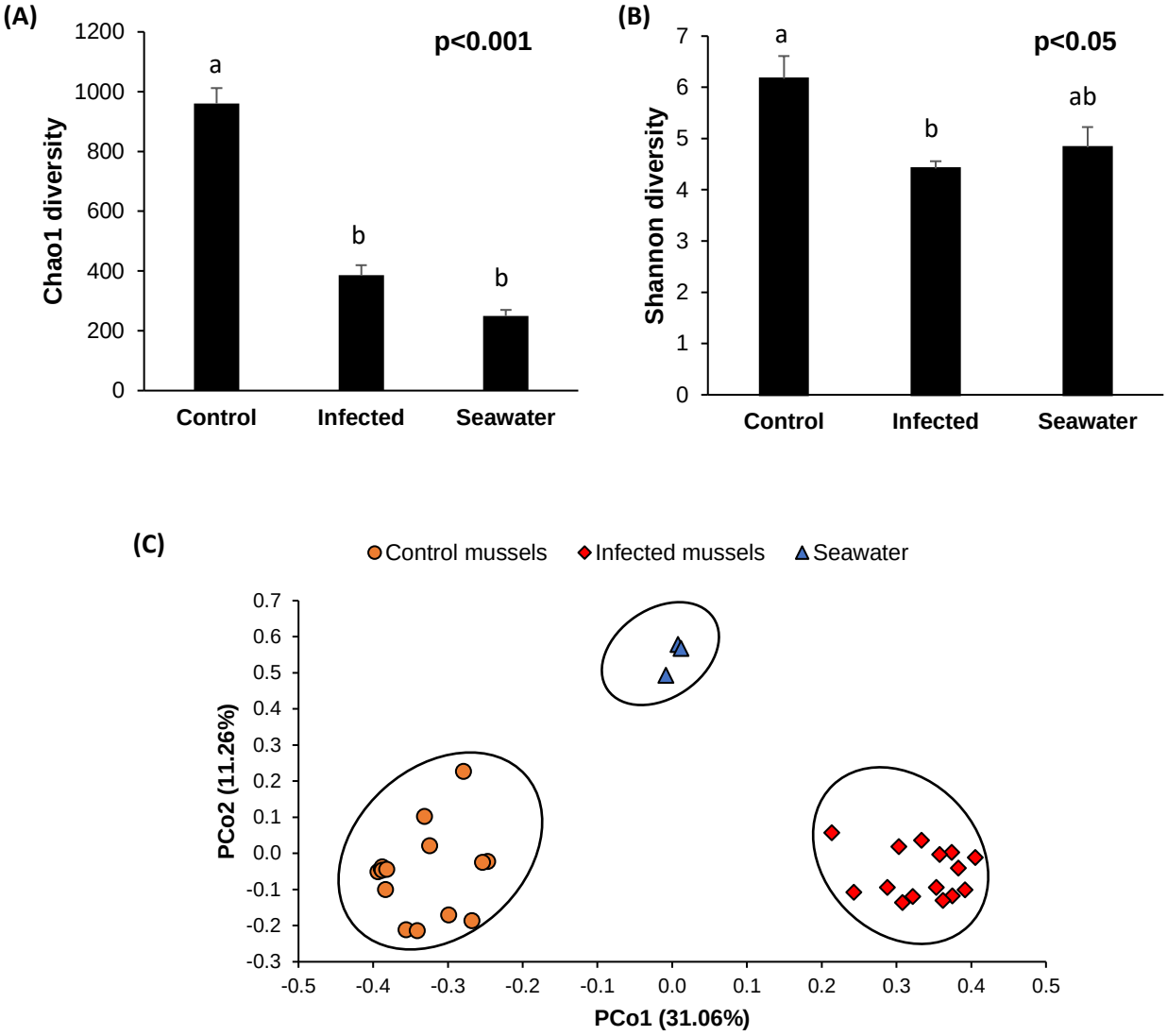
3. Detection of GFP *V. splendidus* in sampled mussels by qPCR

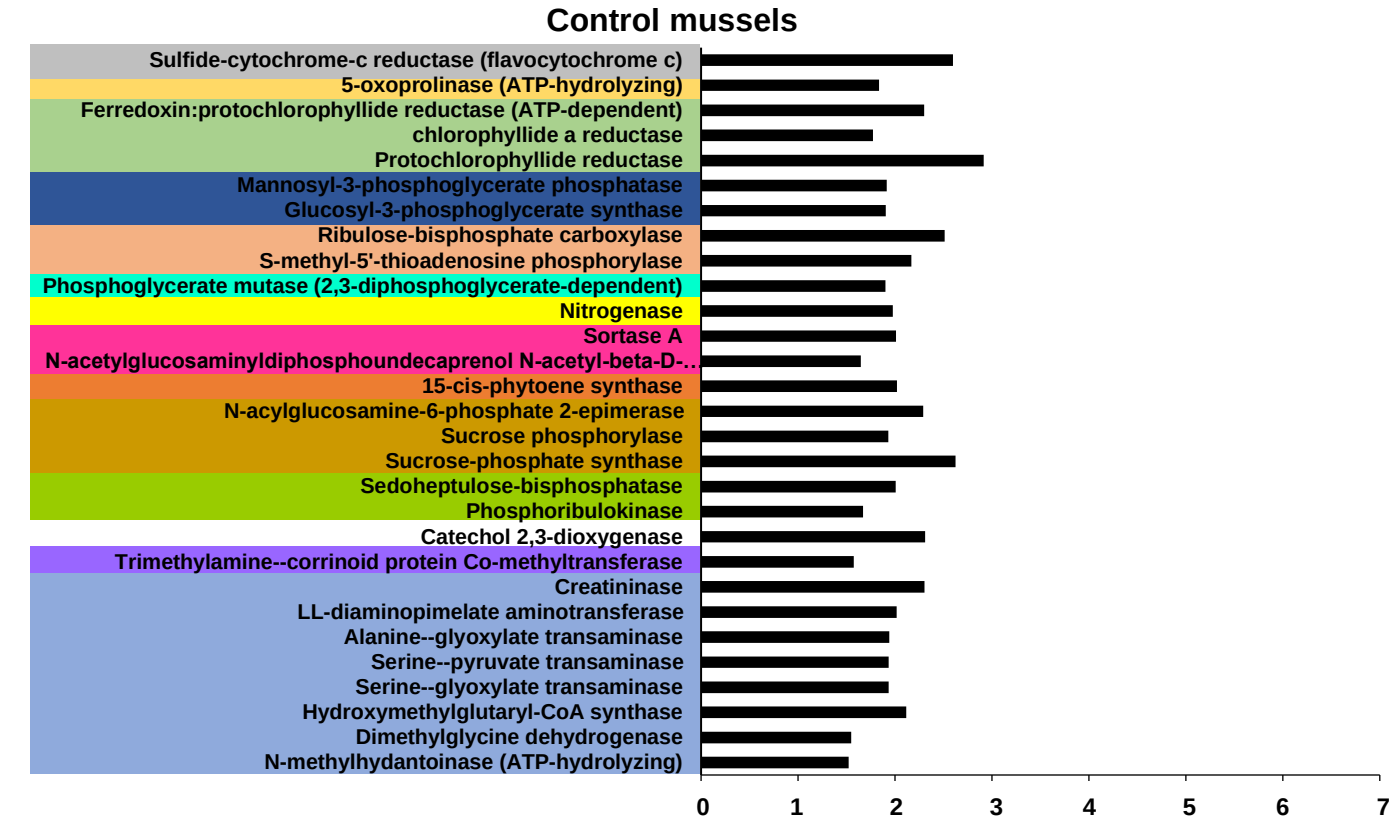


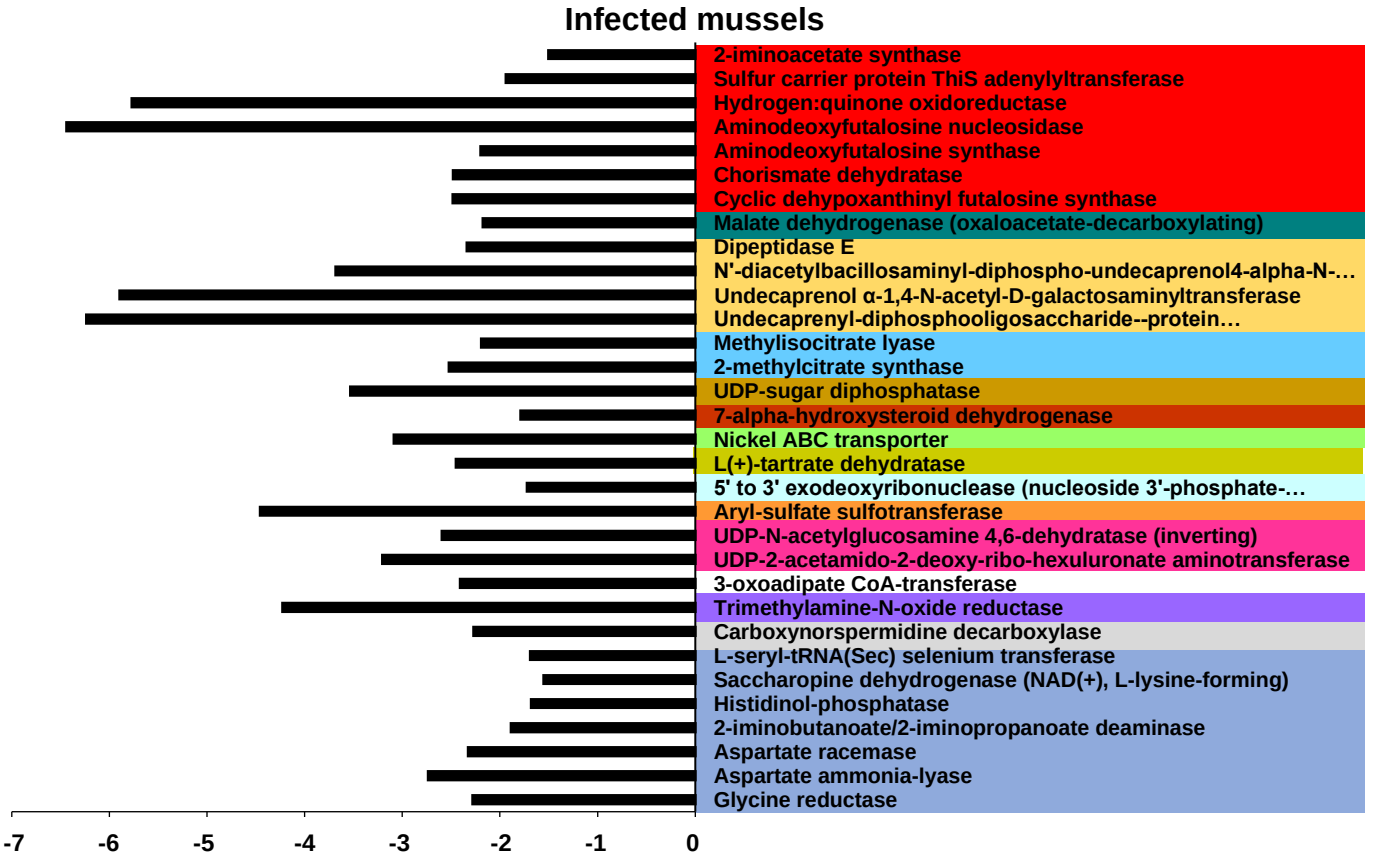
4. 16 S rRNA metabarcoding



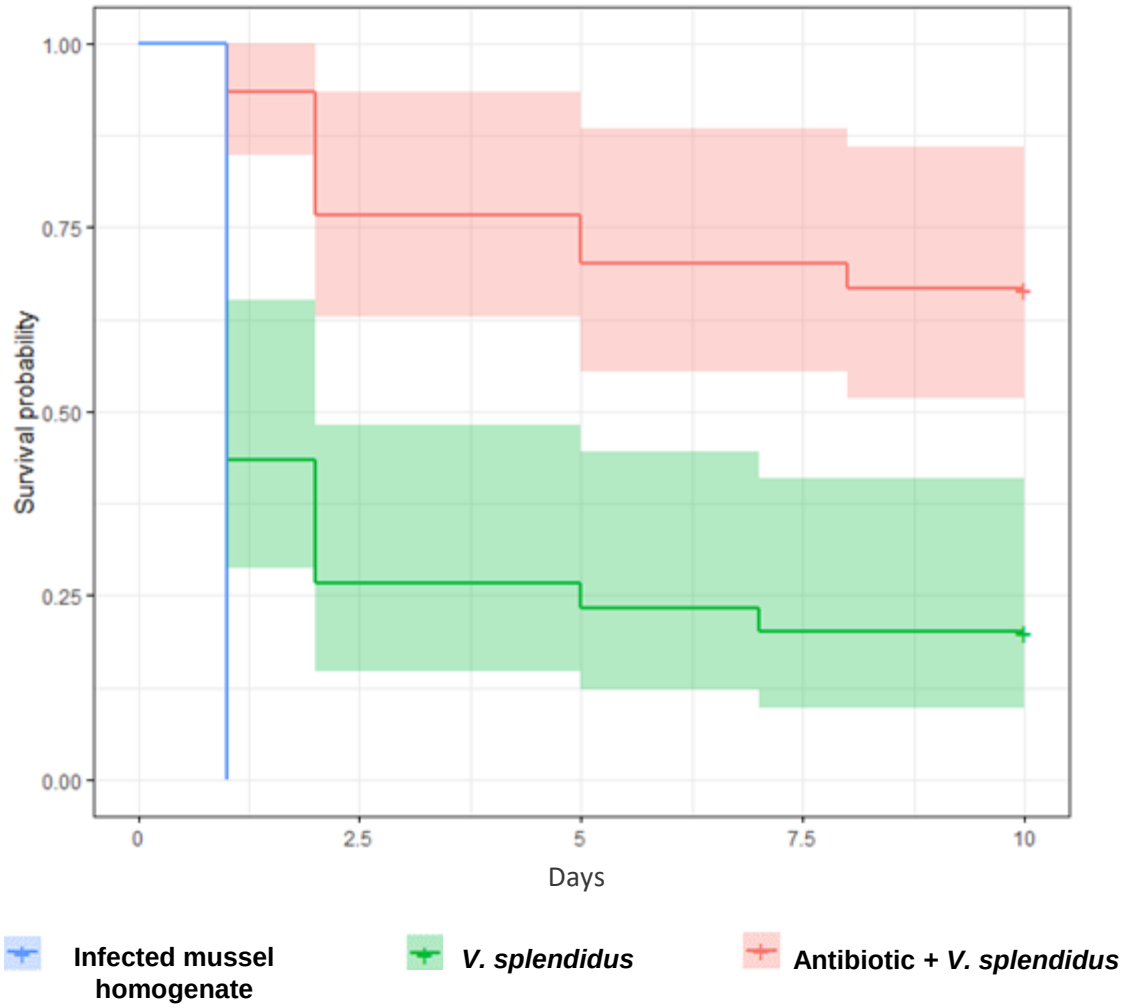

M. edulis control
M. edulis infected
Seawater
(A)
(B)
(C)
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3
Acidobacteria
Actinobacteria
Bacteroidetes
Candidatus Saccharibacteria
Chlamydiae
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Dictyoglomi
Firmicutes
Fusobacteria
Lentisphaerae
Others
Planctomycetes
Proteobacteria
Spirochaetes
Tenericutes
Verrucomicrobia
(D)
(E)
(F)
Acidimicrobiaceae
Flavobacteriaceae
Caldilineaceae
UC Cyanobacteria
Synechococcaceae
Prochloraceae
Leuconostocaceae
Staphylococcaceae
Clostridiaceae
Peptostreptococcaceae
Bacillaceae
Planctomycetaceae
Rickettsiaceae
Rhodobacteraceae
UC Gammaproteobacteria
UC Alphaproteobacteria
Vibrionaceae
Hyphomicrobiaceae
Phyllobacteriaceae
Comamonadaceae
Moraxellaceae
Sphingomonadaceae
UC Rhizobiales
UC Deltaproteobacteria
Halieaceae
Thioalkalispiraceae
UC Spirochaetia
Mycoplasmataceae
Verrucomicrobiaceae
Rubritaleaceae
Marinilabiliaceae
Bacteroidaceae
Marinifilaceae
Porphyromonadaceae
Clostridiaceae
Fusobacteriaceae
UC Epsilonproteobacteria
Moraxellaceae
Shewanellaceae
Campylobacteraceae
Pseudomonadaceae
UC Alteromonadales
Comamonadaceae
UC Alphaproteobacteria
Aeromonadaceae
Colwelliaceae
UC Deltaproteobacteria
Phyllobacteriaceae
Sphingomonadaceae
Mycoplasmataceae
Verrucomicrobiaceae
Rubritaleaceae
-3 -1 1 3
-1 0 1
(G)
(H)
(I)
Verrucomicrobia
Actinobacteria
Bacteroidetes
Tenericutes
Cyanobacteria
Firmicutes
Planctomycetes
Proteobacteria
Ilumatobacter
UC Cyanobacteria
Weissella
Clostridium
Pirellula
Candidatus Megaira
UC Alphaproteobacteria
Labrenzia
Albirhodobacter
Rubritalea
Lutimonas
Synechococcus
Staphylococcus
Blastopirellula
Planctomyces
UC Gammaproteobacteria
Vibrio
Acinetobacter
Mycoplasma
Roseibacillus
Mangroviflexus
Bacteroides
Marinifilum
UC Cyanobacteria
Aeromonas
Motilimonas
UC Alphaproteobacteria
Pseudomonas
Candidatus Megaira
UC Epsilonproteobacteria
Flavobacterium
Carboxylicivirga
Saccharicrinis
Psychrilyobacter
Acinetobacter
Shewanella
Arcobacter
UC Gammaproteobacteria
Vibrio
Mycoplasma
Flavobacterium
Salinirepens
Acinetobacter
Pseudomonas
Aeromonas
Falsirhodobacter
Rheinheimera
Pseudorhodobacter
Shewanella
Hydrogenophaga
Chryseobacterium
Acetobacteroides
Brevundimonas
Aquaspirillum
Malikia
Arcobacter
UC Betaproteobacteria
Novispirillum
Gemmobacter
Comamonas

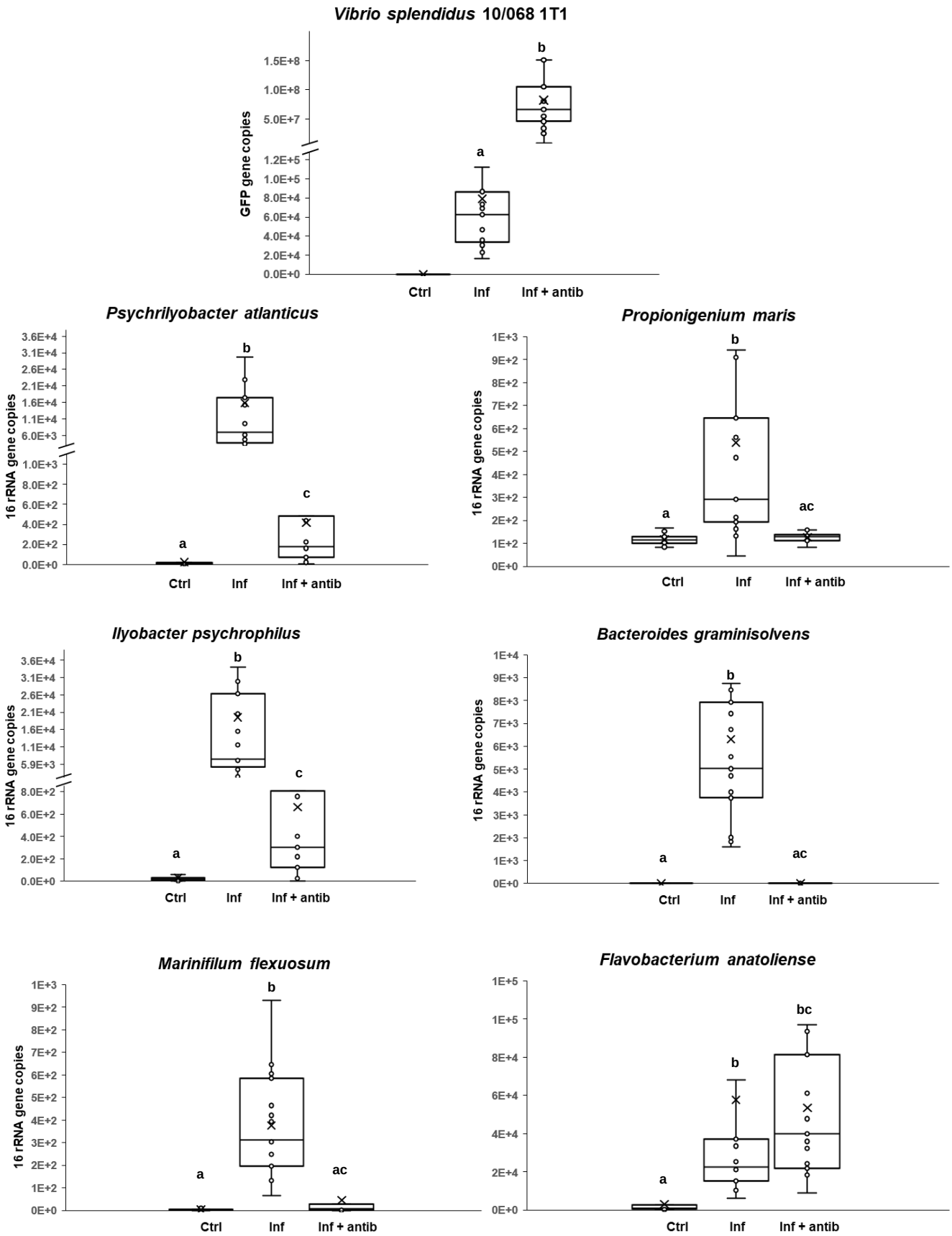

(A)
p<0.001
Chao1 diversity
1200
1000
800
600
400
200
0
a
b
b
Control
Infected
Seawater
(B)
p<0.05
Shannon diversity
7
6
5
4
3
2
1
0
a
b
ab
Control
Infected
Seawater
(C)
Control mussels
Infected mussels
Seawater
PCo2 (11.26%)
PCo1 (31.06%)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5





- Amino acid metabolism
- Amine and polyamine biosynthesis
- Anaerobic respiration
- Aromatic compound degradation
- Autotrophic CO2 fixation
- Carbohydrate metabolism
- Carotenoid biosynthesis
- Cell wall biosynthesis
- Conjugation of compounds
- DNA metabolism
- Nitrogen metabolism
- Glycolysis
- Glyoxylate and dicarboxylate metabolism
- Ion transport
- Nucleoside and nucleotide degradation
- Organic solute biosynthesis
- Porphyrin and chlorophyll metabolism
- Propanoate metabolism
- Protein metabolism
- Sulfure metabolism
- TCA cycle
- Vitamin metabolism
- Bile acids metabolism


1.00
0.75
0.50
0.25
0.00
Survival probability
0
2.5
5
7.5
10
Days
Infected mussel homogenate
V. splendidus
Antibiotic + V. splendidus


Vibrio splendidus 10/068 1T1
GFP gene copies
1.5E+8
1.0E+8
5.0E+7
1.2E+5
1.0E+5
8.0E+4
6.0E+4
4.0E+4
2.0E+4
0.0E+0
a
b
Ctrl
Inf
Inf + antib
Psychrilyobacter atlanticus
16 rRNA gene copies
3.6E+4
3.1E+4
2.6E+4
2.1E+4
1.6E+4
1.1E+4
6.0E+3
1.0E+3
8.0E+2
6.0E+2
4.0E+2
2.0E+2
0.0E+0
a
b
c
Ctrl
Inf
Inf + antib
Propionigenium maris
16 rRNA gene copies
1E+3
9E+2
8E+2
7E+2
6E+2
5E+2
4E+2
3E+2
2E+2
1E+2
0E+0
a
b
ac
Ctrl
Inf
Inf + antib
Ilyobacter psychrophilus
16 rRNA gene copies
3.6E+4
3.1E+4
2.6E+4
2.1E+4
1.6E+4
1.1E+4
5.9E+3
8.0E+2
6.0E+2
4.0E+2
2.0E+2
0.0E+0
a
b
c
Ctrl
Inf
Inf + antib
Bacteroides graminisolvens
16 rRNA gene copies
1E+4
9E+3
8E+3
7E+3
6E+3
5E+3
4E+3
3E+3
2E+3
1E+3
0E+0
a
b
ac
Ctrl
Inf
Inf + antib
Marinifilum flexuosum
16 rRNA gene copies
1E+3
9E+2
8E+2
7E+2
6E+2
5E+2
4E+2
3E+2
2E+2
1E+2
0E+0
a
b
ac
Ctrl
Inf
Inf + antib
Flavobacterium anatoliense
1E+5
1E+5
8E+4
6E+4
4E+4
2E+4
0E+0
a
b
bc
Ctrl
Inf
Inf + antib