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1 **Impact of epizootics on mussel farms: Insights into microbiota composition of *Mytilus***  
2 **species**

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13

14 **Abstract**

15 Outbreaks of marine mussel mortality on French farms could have different aetiologies. One of  
16 them implies *Vibrio splendidus* strains. Beyond the involvement of this pathogen, there is  
17 considerable evidence that diseases often result from interactions between several microbes and  
18 the host. In this study, we explored the bacterial communities associated with mussel species  
19 and the surrounding water collected from a mussel farm affected by mortalities. The microbiota  
20 of *Mytilus edulis*, *Mytilus galloprovincialis* and their hybrids displayed an abnormal abundance  
21 of Proteobacteria, in particular the genera *Vibrio*, *Cobetia* and *Arcobacter*. Despite the  
22 dysbiosis, the Mediterranean mussel showed a different microbiota profile with a higher  
23 richness and presence of the phylum Bacteroidetes. Bipartite network analyses at the level of  
24 bacteria families confirmed this finding and showed that the microbiomes of *M. edulis* and the  
25 hybrids tended to cluster together. In addition, injection of mussels with the virulent *V.*  
26 *splendidus* induced less mortality rate in *M. galloprovincialis* compared to the other *Mytilus* sp.  
27 suggesting a better resistance of the Mediterranean mussel to infection.

28 Our findings point to a probable aetiology of pathobiome-mediated disease in mussels. To fully  
29 understand this phenomenon, more knowledge is needed on the roles of pathobiotic systems  
30 and their development during disease establishment.

31 **Keywords:** Pathobiome, mussel mortalities, opportunistic bacteria, core microbiome

## 32        **1. Introduction**

33        Over the last two decades, European mussel production has been declining. Among the putative  
34        causes of this trend, the spread of diseases, algal blooms, lack of spat, predation and low  
35        profitability have been mentioned (Avdelas et al., 2021). Indeed, since 2014, mass mortality  
36        events have been recorded in blue mussel stocks in France (Bechemin et al., 2014) and since  
37        2016 in the Netherlands (Capelle et al., 2021). While no cause could explain this phenomena in  
38        the Netherlands (Capelle et al., 2021), the involvement of pathogens in mortalities has been  
39        demonstrated in French mussel farms (Ben Cheikh et al., 2017, 2016; Benabdelmouna et al.,  
40        2018; Oden et al., 2016).

41        As filter-feeding organisms, marine mussels are constantly exposed to a variety of  
42        microorganisms including pathogenic bacteria that can threaten their lives. In addition to  
43        external microbes, like all macroorganisms, mussels interact with the microorganisms they  
44        harbour, i.e. their microbiota. This dialogue between the host and its microbiota can help  
45        maintain their respective integrity, as shown e.g. in leeches (Kikuchi and Graf, 2007; Tasiemski  
46        et al., 2015). In fact, advances in analytical techniques over the last few decades have favoured  
47        the expansion of studies on microbial communities, particularly in the human model,  
48        highlighting their importance for their host (Amato, 2016; Bäckhed et al., 2005; Martin et al.,  
49        2009; Mushegian and Ebert, 2016). Considered the “last organ” in humans (Baquero and  
50        Nombela, 2012), a large body of evidence has revealed the beneficial contribution of the  
51        microbiota to basic biological processes such as digestive, metabolic and immune functions  
52        (Callens et al., 2016; Sirisinha, 2016; Wang et al., 2017) and its involvement in the eco-  
53        evolutionary dynamics of their host (Amato, 2016; Kohl et al., 2018; Macke et al., 2017;  
54        Moeller et al., 2016; Murall et al., 2017). However, like other ecosystems, the composition of  
55        the microbiota can be modulated by several factors such as antibiotics and pathogens leading  
56        to an altered community structure or dysbiosis (Clark et al., 2015; Costello et al., 2012; Lamont

57 and Hajishengallis, 2015; Martin et al., 2009). In case of dysbiosis, the mutual harmony  
58 between bacteria is disrupted and the healthy balanced microbiota turns into a disease-inducing  
59 pathobiota (Pitlik and Koren, 2017; Tungland, 2018; Wang et al., 2017).

60 The large number of findings in the human model has sparked research interest in other  
61 organisms including bivalves. Numerous studies have investigated microbiota structure in  
62 oysters, mussels and to a lesser extent clams, under the influence of factors such as host  
63 genotype, environment and pathogens (for review see Paillard et al., 2022). The structure of the  
64 microbiota can be divided into at least two types of communities, namely a stable community  
65 of resident bacteria within a host population (“the core microbiome”) and a variable microbial  
66 community that depends on the surrounding environment (Santibáñez et al., 2022). Data on the  
67 role of the core microbiome are limited, although some authors suggest a contribution to  
68 essential host biological functions (Lemanceau et al., 2017). Furthermore, the balance between  
69 the core and variable microbiome is still unclear, especially as the dynamics of host-associated  
70 communities are directly affected by abiotic and biotic factors. For example, elevated water  
71 temperature appears to be a major determinant and strongly influences the composition of the  
72 microbiota in mussels and oysters by also favouring pathogens such as *Vibrio* sp. (Li et al.,  
73 2019, 2018; Lokmer and Wegner, 2015). In addition, water pollution by nanoparticles,  
74 nanoplastics or chemicals can affect the diversity of the microbiota as has been demonstrated  
75 in *Mytilus galloprovincialis* (Auguste et al., 2020, 2019; Balbi et al., 2020). Besides abiotic  
76 factors, microbiota imbalance has been linked to diseases (King et al., 2019). Indeed, infection  
77 of *Crassostrea gigas* by OsHV-1 virus induced dysbiosis with subsequent bacteraemia by  
78 opportunistic bacteria (Lorgeril et al., 2018). Similarly, field studies on oysters affected by  
79 mortalities have shown signs of microbiota disruption (Lasa et al., 2019).

80 For mussels, only a few studies have explored microbiota dynamics in interaction with  
81 pathogens. Li et al. (2019) reported the reduction in microbial diversity of *M. coruscus*

82 haemolymph after exposure to *Vibrio cyclitrophicus*. Likewise, we recently demonstrated that  
83 experimental infection with *V. splendidus* reduces the diversity of the *M. edulis* microbiota and  
84 favours pathobiontic bacteria (Ben Cheikh and Travers, 2022). However, the response of the  
85 mussel microbiota during disease outbreaks remains unknown.

86 The aim of this study is to provide new insights into the microbiota of mussels experiencing  
87 mortality outbreaks on French farms. For this purpose, the bacterial communities associated  
88 with *M. edulis*, *M. galloprovincialis* and their hybrids, grown in the same environment, and the  
89 surrounding water were analysed. The differences between the microbial communities found in  
90 the different species were assessed using ecological network methods to cluster microbiota and  
91 link the obtained classification to the type of sampled mussels. The susceptibility of the  
92 different types of mussels to infection was then explored by experimental infection with *V.*  
93 *splendidus* strains.

## 94 **2. Material and methods**

### 95 **2.1. Mussel collection**

96 45 farmed adults of *Mytilus* sp. (*M. edulis*, *M. galloprovincialis* and hybrid *M. edulis* x  
97 *galloprovincialis*, 15 individuals from each taxon, size between 4 and 5 cm) were collected  
98 from a mussel farm affected by massive mortalities (Bay of Lannion, France 48°44 44.8 N,  
99 3°35 24.9 W) in September 2018 (mean temperature = 17°C). The animals were immediately  
100 transported to the laboratory and randomly dispatched in different temperature-controlled  
101 (16°C) aerated water tanks filled with seawater sampled from the corresponding site (salinity =  
102 35 PSU). Mussels were taken at random from two different tanks for microbiota analysis or for  
103 experimental infection. Seawater from the same site was also sampled for microbiota analyses.

## 104        **2.2. Microbiota analysis**

105        After cleaning, mussels were removed from their shells, flash frozen in liquid nitrogen and  
106        stored at -80°C (15 individuals/species). Serial filtrations of 5L of seawater were performed  
107        using 8 µm and 5 µm porosity (Whatman™ Nucleopore™ Polycarbonate membrane filter,  
108        47 mm diameter, UK). The resulting filtered seawater was centrifuged for 15 min at 11000 g.  
109        The membranes and bacterial pellets were stored at -80°C.

110        DNA extraction, amplicon library construction and Illumina MiSeq sequencing were performed  
111        by Biofidal (Vaulx-en-Velin, France). DNA was extracted from frozen mussels ground in liquid  
112        nitrogen and from bacteria pellet using Mag Bind® Universal Pathogen DNA Kit (Omega BIO  
113        TEK, Georgia, USA) and from the different filters using Quick DNA/RNA MagBead kit (Zymo  
114        Research, California, USA) according to the manufacturer's protocol. DNA concentration and  
115        quality were checked with the QuantiFluor® dsDNA System (Promega, France). The 16S  
116        rRNA gene of bacterial communities was amplified using the 5X HOT BIOAmp ®  
117        BlendMaster Mix (Biofidal) and the 341F: CCTACGGGNGGCWGCAG (17 pb) and 805R:  
118        GACTACHVGGGTATCTAATCC (21 pb) primers (designed by Biofidal) targeting the  
119        variable V3-V4 loops (Klindworth et al., 2013). After purification of the PCR products by  
120        SPRIselect reagent kit (Beckman Coulter), a second amplification was performed using index  
121        adapter oligos (P5/P7). The purified PCR products were normalized to obtain an equimolar  
122        library and subsequently pooled. Negative controls and ZymoBIOMICS microbial community  
123        standards were included during the library preparation (Supplemental Figure S1). Amplicons  
124        were sequenced on the MiSeq platform with V3 reagents producing 2 X 300 bp paired-end  
125        reads.

126        After demultiplexing, primer sequence adapters were trimmed from the reads using cutadapt  
127        software version 1.12 (Martin, 2011). Paired-end sequence reads were collapsed into so-called  
128        pseudoreads using sequence overlap with USEARCH version 9.2 (Edgar, 2010). After chimera

129 and singletons removing, filtered pseudoreads were aligned to the RDP database version 11.5  
130 (Cole et al., 2014) with Snap-aligner version 1.0 beta.23 (Zaharia et al., 2011) to identify the  
131 taxonomy. Finally, USEARCH was used to create the OTU-table and to calculate alpha  
132 diversity. Principal coordinate analyses (PCoA) were performed via xlstat using the Bray–  
133 Curtis distance matrix. All FastQ files were deposited in SRA-NCBI under the project accession  
134 number PRJNA923705.

### 135 **2.3. Host-microbiota network**

136 To analyse the structure of the host-microbiota association dataset, we used methods borrowed  
137 from the analysis of bipartite ecological networks. First, we aggregated data on microbial taxa  
138 at the family level and transformed the microbial family abundance table into presence/absence  
139 data (i.e., all abundances  $> 0$  were set to 1). We then modelled the obtained bipartite network  
140 of hosts by microbial families using a latent block model (LBM) (Govaert and Nadif, 2008;  
141 Leger et al., 2015). The clustering obtained with LBMs is indeed more useful than the one  
142 obtained with modularity-search algorithms (Newman, 2006; Traag et al., 2019) as blocks can  
143 identify densely connected as well as sparsely connected regions of the network. We tested the  
144 correlation between the obtained clustering of hosts and their classification among species using  
145 a method described in Massol et al. (2021): we computed the normalised mutual information  
146 (NMI) index between the two classifications of the hosts (by host type and by LBM block); we  
147 tested the significance of NMI using randomizations of the bipartite network through the  
148 configuration model (Strona et al., 2014).

### 149 **2.4. Experimental infection**

150 Two strains of *V. splendidus*, the virulent 10/068 1T1 and the non-virulent 12/056 M24 T1 were  
151 used for experimental infection by injection as described in previous studies (Ben Cheikh et al.,  
152 2017, 2016). Briefly, bacteria were grown overnight in Luria Bertani NaCl 20 g.L<sup>-1</sup> at 22°C



153 with constant agitation (80 rpm) and resuspended in filtered sterile seawater (FSSW,  $OD_{600nm}$   
154 = 1,  $2.10^8$  CFU  $mL^{-1}$ ) after double washing (FSWW) /centrifugation (1200 g for 10 min).  
155 Anesthetized mussels (2–3 h in magnesium chloride solution at 50  $g.L^{-1}$ , 1/4: v/v  
156 seawater/freshwater) were intramuscularly injected with 100  $\mu L$  of bacterial suspension or  
157 FSSW for negative controls. The animals were transferred to tanks (3 replicate tanks, 10  
158 mussels per tank) filled with 2 L of UV-treated and filtered seawater supplemented with 50 mL  
159 of phytoplankton (*Isochrysis galbana*) and maintained under static conditions at 16°C with  
160 aeration. Mortality was monitored over 8 days with removing of newly dead mussels from the  
161 tanks.

## 162 **2.5. Statistical analyses**

163 SigmaPlot 12 (Systat Software Inc., Chicago, IL) and R version 4.0.5 (R Core Team, 2021)  
164 were used for statistical analysis. A comparison of OTUs between mussel types and/or seawater  
165 was performed. Values were tested for normality (Shapiro-Wilk test) then a one-way ANOVA  
166 was performed. Pairwise comparisons between groups were assessed by post-hoc analyses  
167 (with Holm-Sidak correction). Statistical significance was accepted for  $p < 0.05$ .

## 168 **3. Results**

### 169 **3.1. Microbial structure in mussels and seawater**

170 The 48 samples (15 *M. edulis*, 15 *M. galloprovincialis*, 15 hybrid and 3 seawater samples)  
171 sequenced using the 16S rRNA gene generated 4 842 875 raw reads ranging from 70 302 to  
172 126 377 reads per sample with an average of 100 893 reads (Supplemental Table S1). After  
173 Chimera filtering, unique pseudoreads averaged 38 597 reads.

174 The clustering of reads in OTUs revealed a higher richness in the Mediterranean mussel *M.*  
175 *galloprovincialis* (significant difference with *M. edulis*, ANOVA  $p < 0.05$ ) and in seawater with  
176 a mean absolute abundance of  $80\,486 \pm 3\,214$  OTUs and  $80\,367 \pm 808$  OTUs respectively. The

177 blue mussel *M. edulis* and the hybrids had a mean absolute abundance of  $68\,068 \pm 2\,322$  and  
178  $70\,637 \pm 2\,783$  OTUs respectively (Figure 1 A). Alpha diversity metrics were higher in *M.*  
179 *galloprovincialis* compared to the other two mussel species and seawater (non-significant data,  
180 supplemental Figure S2A). Beta diversity analysis revealed dissimilarities between mussels and  
181 seawater (Supplemental Figure S2B). The compositional structure at the phylum level indicated  
182 a similarity between *M. edulis* and the hybrids (Figure 1 B) with a predominance of  
183 Proteobacteria (relative abundance r.a. of 92.3% to 99.4% in *M. edulis* and 89.9% to 99.9% in  
184 hybrids) and the presence of Bacteroidetes (r.a. from 0.3% to 5.5% in *M. edulis* and from 0 to  
185 5.4% in hybrids). Other rare phyla with an abundance  $> 1$  were detected in some hybrids, e.g.,  
186 Firmicutes (replicates 2, 6 and 7) and Tenericutes (replicates 1 and 13). Mediterranean mussels  
187 harboured a lower proportion of Proteobacteria (r.a. from 47.3% to 99.6%) and a higher  
188 proportion of Bacteroidetes (r.a. from 0 to 23.5%). OTUs belonging to Tenericutes,  
189 Verrucomicrobia, Fusobacteria and Cyanobacteria were also identified in some mussels with  
190 r.a.  $> 1$ . The composition of the surrounding seawater was similar to that of the mussels with a  
191 notable r.a. of Bacteroidetes (mean  $21.1\% \pm 6.4\%$ ) and the predominance of Proteobacteria  
192 (mean  $77.4\% \pm 6.9\%$ ).

193 A comparative analysis of the most abundant families (Top 30) and genera revealed a more  
194 specific layout for each group although a similar bacterial community was detected in most  
195 samples (Figure 2, Supplemental Table S2). Overall, Vibrionaceae, Halomonadaceae,  
196 Pseudoalteromonadaceae and Campylobacteraceae were the dominant families in the blue  
197 mussel and hybrids (mean r.a. 5-35%) with notably the genera *Vibrio*, *Cobetia*,  
198 *Pseudoalteromonas* and *Arcobacter* but also unclassified Epsilonproteobacteria. Similarly, the  
199 Mediterranean mussel harboured Vibrionaceae (*Vibrio*), Pseudoalteromonadaceae  
200 (*Pseudoalteromonas*) and unclassified Epsilonproteobacteria but also a higher proportion of  
201 Campylobacteraceae (*Arcobacter*) and Flavobacteriaceae (especially *Tenacibaculum* and

202 *Flavobacterium*) (mean r.a. 18.2% and 6.8% respectively). In contrast, in addition to  
203 unclassified Gammaproteobacteria, the prevailing taxa (r.a. up to 5%) in seawater samples were  
204 the families Rhodobacteraceae, Oceanospirillaceae, Pseudoalteromonadaceae,  
205 Crocinitomicaceae and Flavobacteriaceae and the genera *Sulfitobacter*, *Oceanospirillum*,  
206 *Pseudoalteromonas*. The majority of *Vibrio* identified in mussels have been affiliated to the *V.*  
207 *Splendidus* group (Supplemental Table S3).

### 208 **3.2. Association between microbial family structure and mussel taxa**

209 The LBM inferred 5 clusters of hosts and 4 clusters of bacterial families (Figure 3A).  
210 Assignment of host individuals to clusters resulted in probabilities of membership at least as  
211 high as 88.3%; by contrast, a few bacterial families were only assigned to a particular cluster  
212 with a probability of 50.8% (Supplemental Figure S3). The first cluster of hosts consisted in 2  
213 samples, one of *M. galloprovincialis* and one hybrid, which harboured high numbers of  
214 bacterial families (223 families on average). The second cluster of hosts consisted in 20 samples  
215 (1 hybrid, 7 *M. edulis* and 12 *M. galloprovincialis*) with an average of 141.9 bacterial families  
216 per sample. The third cluster included only 4 samples (one replicate of *M. galloprovincialis*  
217 together with the three seawater samples) with an average of 125.5 families per sample. The  
218 fourth cluster of hosts consisted in 16 samples (7 *M. edulis*, 1 *M. galloprovincialis*, 8 hybrids)  
219 with an average of 109 families per sample. The fifth cluster of hosts consisted in 6 samples (1  
220 *M. edulis*, 5 hybrids) with an average of 69 families per sample. The four clusters of bacterial  
221 families harboured 56, 57, 89 and 176 families, respectively, and were found on average in  
222 45.46, 30.44, 13.44 and 2.63 host samples respectively. Probabilities of host-bacterial family  
223 interactions can be found in Supplemental Table S4.

224 The classification of the hosts (48 samples) according to their LBM cluster was significantly  
225 congruent with their classification based on sample type (mussel taxon or seawater sample, i.e.,  
226 4 groups) according to the NMI (NMI = 0.381, p value < 0.0001 when tested against 10,000

227 randomizations of the bipartite network). Indeed, a graphical representation of the two  
228 classifications evince that *M. galloprovincialis* projects almost completely onto LBM cluster 2  
229 while LBM clusters 4 and 5 project mostly onto *M. edulis* and hybrids (Figure 3B). Seawater  
230 samples coincide almost perfectly with LBM cluster 3.

### 231 **3.3. Susceptibility of mussels to pathogens**

232 To assess the susceptibility of mussel populations to bacterial infection, *M. edulis*, *M.*  
233 *galloprovincialis* and hybrids were injected with two strains of *V. splendidus* of contrasting  
234 virulence. Exposure of mussels to avirulent strain 12/056 M24 T1 induced limited mortalities  
235 in all mussel groups, ranging from  $3.3\% \pm 3.3\%$  in hybrids to  $13.3\% \pm 3.3\%$  in *M. edulis* at day  
236 8 (Figure 4). In contrast, injection of the virulent strain 10/068 1T1 induced higher mortalities  
237 with different rates in the mussel species. The first mortalities appeared 24h post-injection and  
238 increased progressively until stabilization on day 8. Hybrids and *M. edulis* had different patterns  
239 at the beginning of the infection ( $16.6\% \pm 12\%$  for *M. edulis* and  $46.6\% \pm 6.6\%$  for hybrids on  
240 day 1) but reached 76.6% of mortality at the end of the experiment while *M. galloprovincialis*  
241 had lower mortalities ( $23.3\% \pm 3.3\%$  on day 1 and  $46.6\% \pm 12\%$  on day 8). No or negligible  
242 mortality was observed in controls.

## 243 **4. Discussion**

244 Since 2014, mass mortalities have been reported in French mussel farms. While *M. edulis*, *M.*  
245 *galloprovincialis* and their hybrids represent the main farmed species, the epizootics seem to  
246 mostly affect the blue mussel (Bechemin et al., 2014). In general, investigations of mussel  
247 disease have so far been limited to the detection and isolation of known pathogens from  
248 moribund animals such as *V. splendidus* (Oden et al., 2016), combined with experimental  
249 infection in the laboratory (Ben Cheikh et al., 2017, 2016). In this study, we provide an  
250 overview of the bacterial communities associated with three mussel species and the surrounding  
251 water sampled from a farm affected by mortality epidemics.

#### 252 **4.1. The microbiota of the three *Mytilus* sp. is dysbiotic**

253 According to previous studies, the microbiota of healthy marine bivalves, and mussels in  
254 particular is characterized by the presence of different phyla with a predominance of  
255 Proteobacteria but also other bacteria belonging to Cyanobacteria, Firmicutes, Planctomycetes,  
256 Bacteroidetes, Actinobacteria, Verrucomicrobia, Tenericutes and Chloroflexi (Ben Cheikh and  
257 Travers, 2022; Musella et al., 2020). Here, the analysis of whole animal microbiota showed a  
258 severe dysbiosis marked by an abnormal dominance of Proteobacteria (Fig. 1, mean r.a. ranging  
259 from 86.5% to 96.1%). Microbiota imbalance has been linked to mortality events and infectious  
260 diseases in bivalves. Field analysis of the Pacific oyster *C. gigas* showed that animals  
261 experiencing mortality outbreaks at different life stages displayed signs of dysbiosis resulting  
262 in a change in the diversity and composition of the microbiota (Lasa et al., 2019). Similarly, a  
263 study on Unionida freshwater mussel during mass mortality events revealed bacterial invasion  
264 and shifts in the bacterial microbiome (Richard et al., 2021). For marine mussels, there are no  
265 data available to our knowledge on animal microbiota during mortality events. However, we  
266 have recently reported a disruption of the blue mussel microbiota during experimental infection  
267 with *V. splendidus* and demonstrated the implication of some opportunistic pathobiontic  
268 bacteria in the infection (Ben Cheikh and Travers, 2022). Thus, even if the causal origin of  
269 mussel mortalities in France still not clear, we can establish a link with pathogenic bacteria,  
270 especially as they are detected in *Mytilus* tissues. Our analysis highlighted the genera *Vibrio*,  
271 *Cobetia* and *Arcobacter* as the predominant bacteria in mussel species (Fig. 2). The abundance  
272 of *Vibrio* sp. in the microbiota of mussels is not surprising as these genera are naturally  
273 occurring in marine environments and certain strains are often associated with outbreaks of  
274 mortality marine bivalves (Travers et al., 2015). In our study, we mainly detected strains  
275 belonging to the *V. Splendidus* clade which includes pathogens of bivalves such as *V.*  
276 *splendidus*, *V. tasmaniensis* and *V. crassostreae* (Ben Cheikh et al., 2016; Bruto et al., 2017;

277 Duperthuy et al., 2011) but also less studied strains showing virulence for some marine  
278 organisms (e.g. *V. toranzoniae* and *V. kanaloae*) and environmental species without pathogenic  
279 potential (e.g. *V. gigantis* and *V. atlanticus*) (Lasa et al., 2017; Romalde et al., 2014). In  
280 accordance with our findings, different studies have reported the presence of *V. splendidus*-  
281 related strains in different French mussel farms affected by mortalities (Bechemin et al., 2014;  
282 Charles et al., 2020a; Oden et al., 2016). However, these studies are restrictive as they are based  
283 on the isolation of culturable bacteria of interest and are therefore not representative of the  
284 animal's microflora. In addition to *V. splendidus* strains, *Francisella haliotica* has been  
285 recently described as potentially involved in blue mussel mortality (Cano et al., 2022; Charles  
286 et al., 2020b). The implication *F. haliotica* in mussel disease is not confirmed in our study  
287 since this strain was detected at very low proportions in some individuals of blue mussels and  
288 hybrids (data not shown). In contrast, metagenomic analyses showed the prevalence of bacteria  
289 of the genus *Cobetia* and *Arcobacter*. While knowledge of *Cobetia* strains is limited, several  
290 reports described the dominance of *Arcobacter* sp. in oysters and mussels in a context of disease  
291 suggesting them as opportunistic bacteria (Ben Cheikh and Travers, 2022; Li et al., 2019;  
292 Lokmer and Wegner, 2015; Lorgeril et al., 2018). Nevertheless, the potential role of these  
293 bacteria needs further investigation, as some species (e.g. *A. cryaerophilus*, *A. ellisii*, *A.*  
294 *nitrofigilis*) have virulence genes and the ability to colonise and enter human cells (Ferreira et  
295 al., 2016; Levican et al., 2013).

#### 296 **4.2. Microbiota of *M. galloprovincialis* differs from *M. edulis* and hybrids**

297 The analysis of microbiomes using a latent block model identified four clusters of bacteria  
298 families and five clusters of host individuals (Fig. 3A). Since this clustering proved to be  
299 congruent with the one obtained from host types, with a significantly high NMI, this suggests  
300 that microbiota are different between mussel species. Indeed, host clusters 4 and 5 belong  
301 almost exclusively to *M. edulis* and hybrids, while *M. galloprovincialis* individuals are almost

302 all found in host cluster 2. The differences in bacteria family richness between clusters thus  
303 confirm that *M. galloprovincialis* is associated to a richer microbiome than both *M. edulis* and  
304 hybrids. The microbial cluster 3 is more associated with host cluster 2 than with host clusters 4  
305 and 5 (probabilities of interactions: 36.6% vs. 18.1% and 8.7%; Supp. Table S4). Therefore, it  
306 probably contains some of the bacterial families peculiar to *M. galloprovincialis*. Despite these  
307 differences, the latent block model also uncovered a potential list of bacterial families that could  
308 constitute the ‘core microbiome’ of all sampled mussels, i.e., microbial cluster 1. This group of  
309 56 bacterial families comprises quite common families such as Aeromonadaceae,  
310 Enterobacteriaceae, Pseudomonadaceae, Rickettsiaceae and Vibrionaceae (Supp. Table S5). In  
311 particular, this cluster contains the three families comprising the three genera described in Fig.  
312 2 (Halomonadaceae, Campylobacteraceae and Vibrionaceae).

313 The shared core microbiota among the three mussel species confirms that the surrounding  
314 environment plays a major role in shaping the composition of the microbiome. In agreement  
315 with our findings, previous reports have shown that the microbiota of co-cultivated oysters and  
316 mussels displayed a high degree of similarity (Pierce and Ward, 2019; Vezzulli et al., 2018).  
317 However, host genetics also appears to be a driving factor in the mussel microbiota given the  
318 dissimilarity between the microbial communities associated with *M. galloprovincialis* and the  
319 other two species. The specific microbiome of the Mediterranean mussel may confer fitness  
320 advantages to the host including resistance and survival from disease as demonstrated in other  
321 organisms (Kueneman et al., 2016; Madison et al., 2022; Zackular et al., 2013). Compared to  
322 *M. edulis* and hybrids, *M. galloprovincialis* showed a lower abundance of *Cobetia* and  
323 *Arcobacter* species. In addition, despite the high prevalence of *V. Splendidus* strains, the  
324 Mediterranean mussel survived experimental infection with the virulent *V. splendidus* 10/068  
325 1T1 better (Fig. 4). Resistance of *M. galloprovincialis* to infectious diseases has been previously  
326 reported. Fuentes et al. (2002) described higher warm season mortality of *M. galloprovincialis*

327 x *M. edulis* hybrids than *M. galloprovincialis* individuals. Similarly, Benabdelmouna et al.  
328 (2018) suggested a higher susceptibility in *M. edulis* relative to *M. galloprovincialis* at the spat  
329 stage, which is consistent with field mortalities. Thus, the microbiome of *M. galloprovincialis*  
330 may be related to the efficiency of defence mechanisms and resistance to infectious diseases.  
331 Characterization of host-microbiota dynamics at the spatial and temporal scales will be crucial  
332 to understanding the roles of the microbiome in maintaining host homeostasis.

## 333 **5. Conclusions**

334 In this study, we provide for the first time an overview of the microbiota of *M. edulis*, *M.*  
335 *galloprovincialis* and their hybrids in the context of mortalities in French mussel farm. We  
336 reported dysbiosis of the microbiota in the three mussel species and a difference between  
337 bacterial communities in seawater and mussel tissues but also between mussel species. Among  
338 the pathobiontic bacteria detected, members of *Vibrio*, *Cobetia* and *Arcobacter* genera were the  
339 most abundant suggesting their involvement in mussel disease. In addition, *M. galloprovincialis*  
340 showed a different microbiota profile and was also more resistant to infection by *V. splendidus*.  
341 Thus, we can speculate on the link between the microbiota of Mediterranean mussels and their  
342 resistance to infectious disease. Further research is needed to understand the nature of the  
343 interactions of mussel microbiota, including the pathobiome, with their host, which may  
344 ultimately help to manage and monitor host health to prevent mortalities in mussel farms.

## 345 **CRedit authorship contribution statement**

346 **Yosra Ben Cheikh:** Conceptualization, Methodology, Validation, Formal analysis,  
347 Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, supervision.

348 **François Massol:** Methodology, Formal analysis, Writing - Original Draft, Writing - Review  
349 & Editing, Visualization. **Nathalie Giusti-Petrucciani:** Investigation. **Marie-Agnès Travers:**  
350 Conceptualization, Writing - Review & Editing.



351 **Data availability statement**

352 The data that support the findings of this study are available from the corresponding author  
353 upon reasonable request.

354 **Declaration of competing interest**

355 The authors declare no conflicts of interest.

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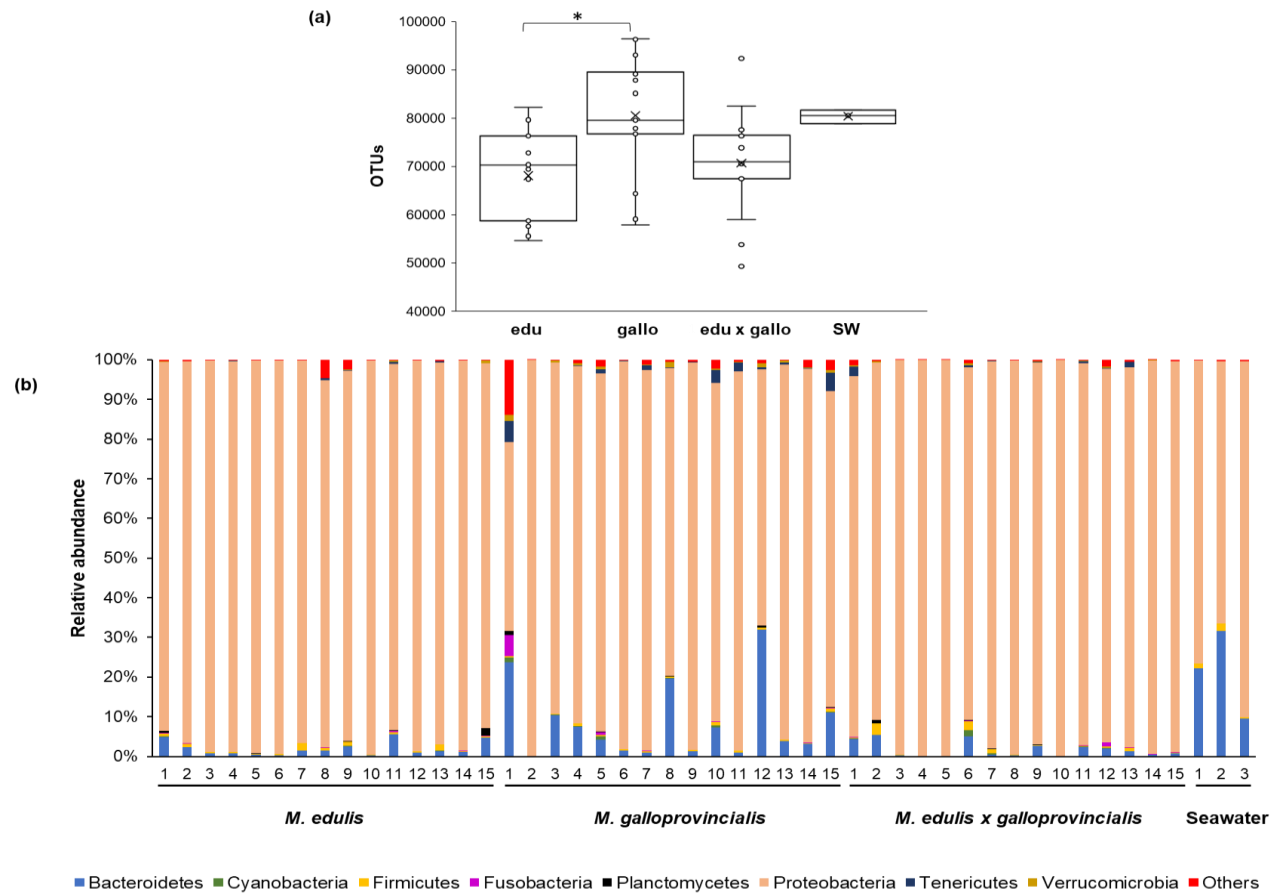
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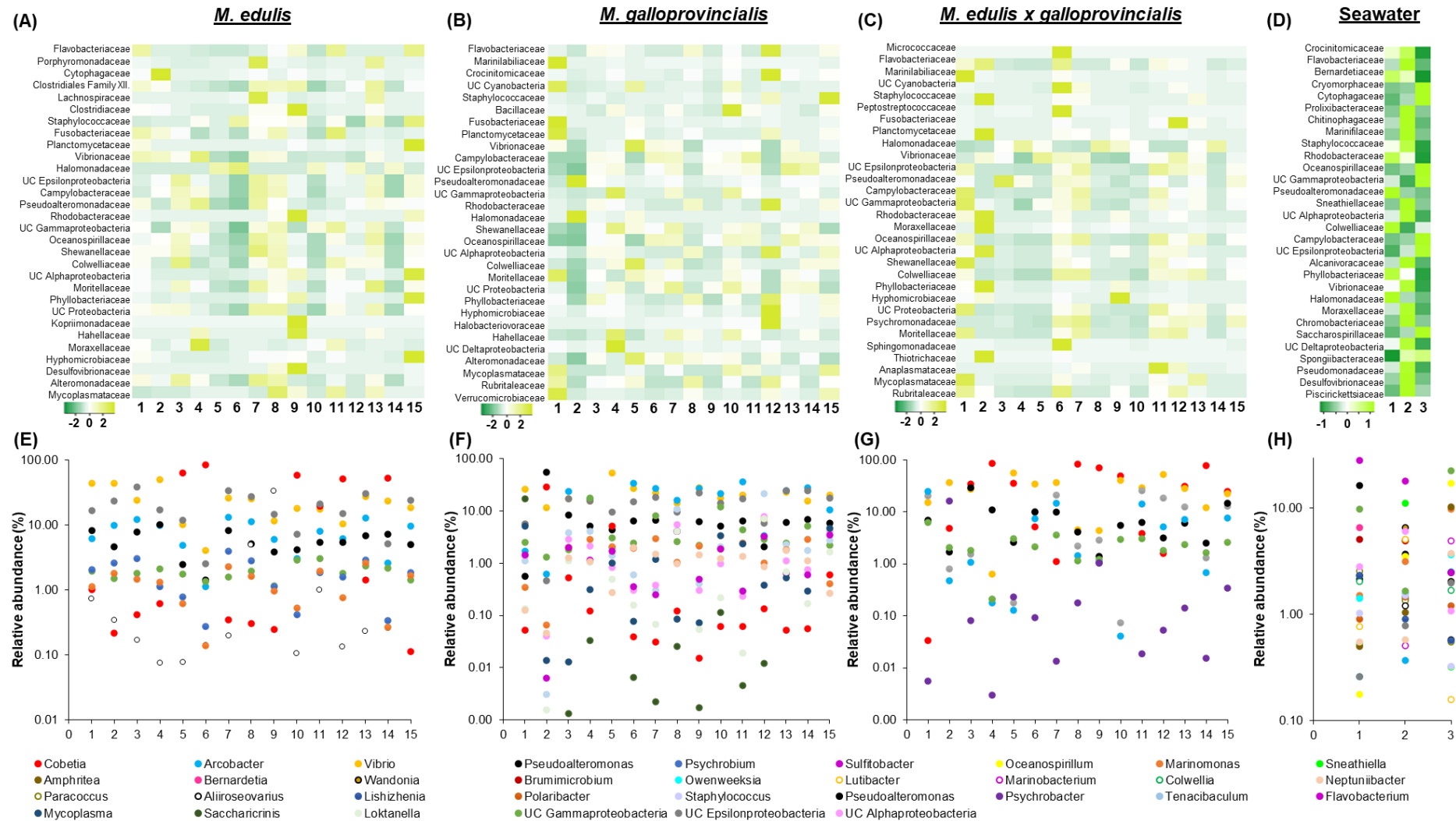
595 **Figure 1. Total OTUs and the relative abundance of bacterial phyla in mussels and seawater.** (A) total OTUs presented by box-and-whisker  
 596 plots showing high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively and the mean  
 597 with a cross. Edu = *M. edulis*, gallo = *M. galloprovincialis*, edu x gallo = *M. edulis* x *galloprovincialis* and SW = seawater. \* indicates significant  
 598 difference  $p < 0.05$  (ANOVA, Holm-Sidak test); (B) relative abundance of bacterial communities at the phylum level. Replicates are labelled with  
 599 the numbers 1 to 15 for animals and 1 to 3 for seawater.





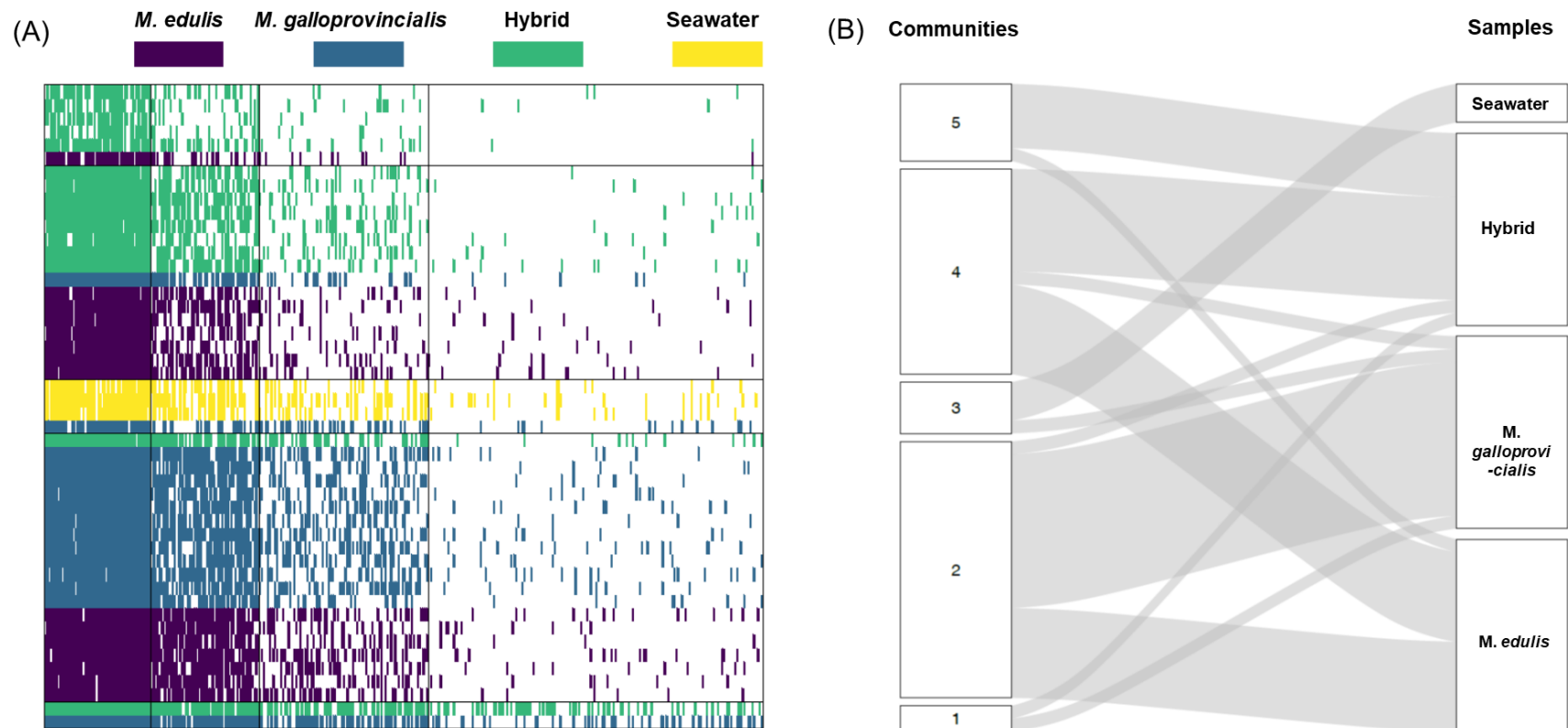
601 **Figure 2. The most abundant bacterial family and genera in *Mytilus* sp. and seawater.** (A-D) heatmaps revealing the top 30 bacterial families  
 602 (%); (E-H) Bacterial genera with a mean relative abundance >1. Replicates are labelled with the numbers 1 to 15 for animals and 1 to 3 for seawater.

603



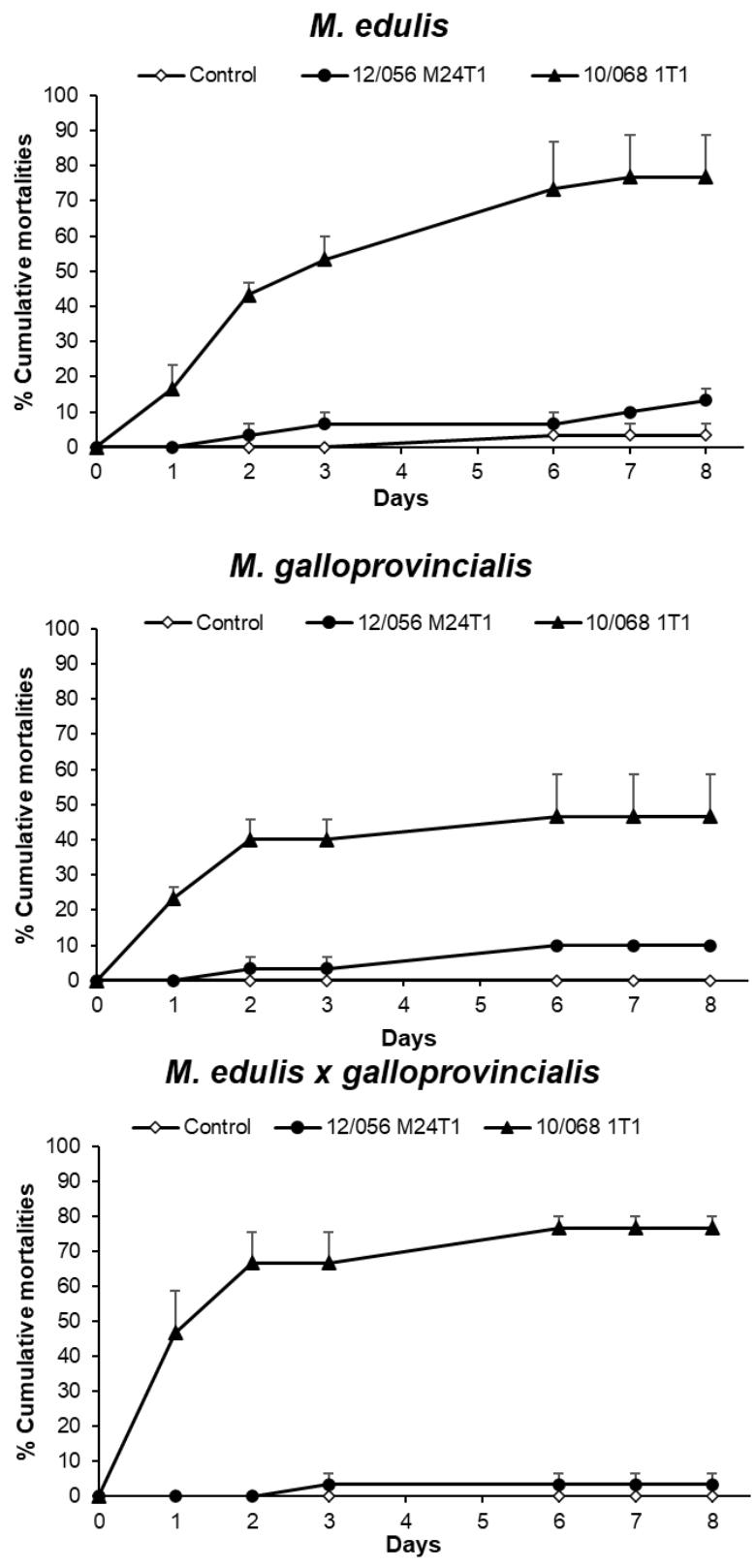
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605 **Figure 3. Clustering of hosts and bacterial families.** (A) Representation of the association between hosts (rows) and bacterial families (columns)  
606 as coloured vs. white rectangles. Colours are associated to the four different host types (purple: *M. edulis*, blue: *M. galloprovincialis*, green: hybrids,  
607 yellow: seawater). The solid black lines (vertical and horizontal) delimit the clustering obtained using the latent block model, e.g. hosts (rows)  
608 enclosed by the same horizontal lines belong to the same host cluster. (B) Alluvial plot linking the individual hosts within the five host clusters  
609 (from cluster 1 at the bottom to cluster 5 at the top, on the left-hand side) with their host type (on the right-hand side).



610

611 **Figure 4. Resistance of *Mytilus* sp. to *V. splendidus* infection.** Mussels were intramuscularly injected with  
 612 the pathogenic *V. splendidus* 10/068 1T1 or with the non-virulent *V. splendidus* 12/056 M24 T1 or with FSSW  
 613 (control). Curves are mean of cumulative mortalities in triplicate tanks (10 mussels/tank)  $\pm$  SEM.



615 **Supplemental Figure S1. Relative abundance of ZymoBIOMICS microbial standards at the genus level.**

616 ZymoBIOMICS kit theoretically contains 8 bacterial strains: *Pseudomonas aeruginosa*, *Escherichia coli*,  
617 *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria*  
618 *monocytogenes*, *Bacillus subtilis*.

619 **Supplemental Figure S2. Alpha and beta diversity in mussels and seawater.** (A) alpha diversity expressed  
620 as Chao1 and Shannon index. Data are the mean  $\pm$  SEM (n=15 for mussels, n=3 for seawater); (B) Beta  
621 diversity calculated using Principal Component Analysis (PCoA) applied on Bray-Curtis distance matrix.

622 **Supplemental Figure S3. Uncertainty of cluster assignments.** Violin plots representing the distribution of  
623 probabilities of assignment of each host (left panel) and bacterial family (right panel) to its LBM cluster (i.e.  
624 the highest among all the probabilities of cluster memberships for each host and family). The white circle  
625 indicates the value of the median of the distribution.

626 **Supplemental Table S1.** Number of pseudoreads in mussels and seawater before and after chimera filtering.

627 **Supplemental Table S2.** Mean relative abundance of the top 30 bacterial families identified in mussels and  
628 seawater, n = 15 for mussels and n = 3 for seawater. Data are percentage  $\pm$  SEM.

629 **Supplemental Table S3.** Mean absolute abundance of *Vibrio*, *Cobetia* and *Arcobacter* species identified in  
630 mussels, n = 15 for mussels. Data are mean  $\pm$  SEM. Only mean abundance > 100 OTUs in at least one mussel  
631 species is considered.

632 **Supplemental Table S4.** Matrix summarizing the estimated probabilities of association between hosts and  
633 bacterial families according to the LBM clusters they belong to. For instance, associations between a random  
634 host from host cluster 3 and a random family from microbial cluster 4 occur with a probability of 7.88%.

635 **Supplemental Table S5.** Inventory of the bacterial families found in each of the four latent block model  
636 clusters for bacteria.

637