Microbiota composition and evenness predict survival rate of oysters confronted to Pacific Oyster Mortality Syndrome

Running title: **Links between mortality and microbiota**

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Abstract

Pacific Oyster Mortality Syndrome (POMS) affects *Crassostrea gigas* oysters worldwide and caused important economic losses. Disease dynamics was recently deciphered and revealed a multiple and progressive infection caused by the *Ostreid herpesvirus* OsHV-1 µVar, triggering an immunosuppression followed by microbiota destabilization and bacteraemia by opportunistic bacterial pathogens. However, it remains unknown if microbiota might participate to oyster protection to POMS, and if microbiota characteristics might be predictive of oyster mortalities. To tackle this issue, we transferred full-sib progenies of resistant and susceptible oyster families from hatchery to the field during a period in favour of POMS. After five days of transplantation, oysters from each family were either sampled for individual microbiota analyses using 16S rRNA gene-metabarcoding or transferred into facilities to record their survival using controlled condition. As expected, all oysters from susceptible families died, and all oysters from the resistant family survived. Quantification of OsHV-1 and bacteria showed that five days of transplantation was long enough to contaminate oysters by POMS, but not for entering the pathogenesis process. Thus, it was possible to compare microbiota characteristics between resistant and susceptible oyster families at the early steps of infection. Strikingly, we found that microbiota evenness and abundances of Cyanobacteria (Subsection III, family I), Mycoplasmataceae, Rhodobacteraceae, and Rhodospirillaceae were significantly different between resistant and susceptible oyster families. We concluded that these microbiota characteristics might predict oyster mortalities.

Introduction

The farmed oyster *Crassostrea gigas* is heavily affected by the Pacific Oyster Mortality Syndrome (POMS) targeting juveniles (Barbosa Solomieu et al., 2015; Pernet et al., 2016). This disease is multifactorial and depends on water temperature (Petton et al., 2015), development stage (Azéma et al., 2017), and oyster diet (Pernet et al., 2019). It is also polymicrobial due to the combined development of viral and bacterial infections (de Lorgeril et al., 2018). Different susceptibility levels were previously associated with oyster physiological status, genetic backgrounds (Dégremont et al., 2005; Samain et al., 2007; Wendling et al., 2017), in association with microbiota dysbiosis (Lokmer and Wegner, 2015).

Recently, holistic molecular approaches revealed the mechanism of POMS (de Lorgeril et al., 2018; Rubio et al., 2019). These studies showed that an infection by the Ostreid herpesvirus (OsHV-1 µVar) is the critical step in the infectious process leading to an immune-compromised state by altering hemocyte physiology. This first process is followed by a microbiota destabilization which "opens the door" to bacterial pathogens (*e.g.*, vibrios) that target hemocytes to induce their lysis. The infectious process is completed with subsequent bacteraemia, which is the ultimate step inducing oyster death.

So far, it is still unknown whether oyster microbial associates might influence disease development, and if microbiota characteristics might predict oyster mortalities. However, microbiota can play a role of physical barriers against pathogens. For example, it was suggested that part of the resident hemolymph bacteria may contribute to oyster protection by producing antimicrobial peptides (Desriac et al., 2014). Other studies highlighted protective effects of (i) secondary endosymbiont of aphids against parasitoid wasps (Oliver et al., 2003), (ii) toxic alkaloids produced by endophytic fungi of grasses against herbivores (Clay, 1990), or (iii) symbiotic bacteria of frogs against pathogenic fungi (Woodhams et al., 2007). Furthermore, microbial associates might also stimulate immunity of their hosts (Wang et al., 2019), and thus indirectly limit pathogen development.

To tackle this issue, we used one resistant oyster family (R_{F21}) and two susceptible families (S_{F15} and S_{F32}) used in a previous study (de Lorgeril et al., 2018). Pathogen-free oysters (reproduced and grown in bio-secured conditions) from these three full-sib families were placed for five days in the field during an infectious period (Figure 1). According to previous observations, 16°C was a relevant threshold to define the infectious period, as high mortality rates were observed above this temperature (Pernet et al., 2012; Petton et al., 2013;

76 Dégremont et al., 2015). Moreover, five days were considered sufficient for oyster
77 contamination by the causal agents of the disease (Petton et al., 2015), and allow microbiota
78 analyses before disease development and animal death. In both the hatchery (control) and
79 after five days of transplantation in the field, oysters were sampled, and we analyzed oyster-
80 associated bacterial communities using 16S rRNA gene-metabarcoding.

81 This study aimed at comparing bacteria content of resistant and susceptible families in order
82 to possibly identify microbiota features associated with oyster mortality and/or resistance.
83 According to a previous holistic study (de Lorgeril et al., 2018), we expected that susceptible
84 families had (i) early microbiota destabilization (increase of species richness, decrease of
85 evenness, increase of microbiota dispersions), and (ii) possibly contained opportunistic and/or
86 pathogenic bacteria (*Vibrio* sp., *Arcobacter* sp., etc.).

87 **Materials and Methods**

88 **Full-sib *C. gigas* oyster families**

89 In 2015, full-sib *C. gigas* oyster families were produced using a methodology that allowed the
90 production of pathogen-free juveniles (de Lorgeril et al., 2018). Two oyster families (S_{F15} and
91 S_{F32}) were produced using one female and one male sampled from wild populations in the
92 Atlantic Ocean and the Mediterranean Sea, respectively (Figure 1). In addition, family R_{F21}
93 was produced using genitors from a mass selective breeding program aiming to increase the
94 resistance of *C. gigas* oysters against OsHV-1. It was performed by breeding disease
95 survivors throughout four generations of selection (Dégremont et al., 2015). Thus, each
96 family (cohort) corresponded to the offspring of a biparental reproduction (full-sib progenies).
97 Conditionning, reproduction and larval breeding were performed as described previously (de
98 Lorgeril et al., 2018). At the larval and post-larval stages, oysters were fed with the same diet
99 as the genitors at a concentration between 1500-2000 μm^3 μl^{-1} (Rico-Villa et al., 2009).
100 Before experiments, all oyster families were maintained in controlled condition at the
101 laboratory (Argenton, France) using seawater treated with UV, filtered through 1 µm mesh,
102 and enriched with a bi-specific phytoplankton diet made of *Tisochrysis lutea* (CCAP 927/14)
103 and *Chaetoceros muelleri* (CCAP 1010/3) (in equal biomass proportion) at a ratio equivalent
104 to 6% of the oyster dry mass (Rico-Villa et al., 2009). Finally, all oysters remained free of any
105 abnormal mortality.

106 **Experimental design**

107 About 140 juveniles per oyster family were either kept in the controlled condition or placed in
108 the field for five days. The field site was located within a oyster farm in the Atlantic Ocean
109 during an infectious period (Figure 1). This infectious period was selected according to
110 seawater temperatures (above 16°C), and was confirmed by the observed mortality rates.
111 After five days of transplantation (July 2016), no mortality occurred and 14 individuals per
112 family were flash frozen in liquid nitrogen and stored at -80°C. The remaining oysters were
113 then transferred into the hatchery under controlled conditions to monitor the survival rates of
114 the three families. The number of dead oysters was recorded at day 13 (*i.e.*, eight days after
115 the end of transplantation). Similarly, 14 individuals per family (except 13 for S_{F32}) kept in
116 the controlled condition were flash frozen in liquid nitrogen and stored at -80°C.

117

118 **DNA extraction, PCR and sequencing**

119 Frozen oysters were ground in liquid nitrogen in 50 ml stainless steel bowls using 20 mm
120 diameter grinding balls (Retsch MM400 mill). The powders were stored at -80°C, and were
121 then used for DNA extractions using the DNA from tissue Macherey-Nagel kit (reference
122 740952.250) according to the manufacturer's protocol. In order to improve DNA extractions,
123 we added a crushing step, which consisted in an additional 12 minutes mechanical lysis using
124 zirconium beads before the 90 min enzymatic lysis in the presence of proteinase K. DNA
125 concentration and quality were checked with Epoch microplate spectrophotometer (BioTek
126 Instruments, Inc.).

127 Then, the 16S rRNA gene of bacterial communities was amplified and sequenced using the
128 variable V3V4 loops (341F: 5'-CCTACGGGNGGCWGCAG-3'; 805R: 5'-
129 GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). Paired-end sequencing
130 (250 bp read length) was performed at the McGill University (Génome Québec Innovation
131 Centre, Montréal, Canada) on the MiSeq system (Illumina) using the v2 chemistry according
132 to the manufacturer's protocol. Raw sequence data are available in the SRA database
133 (BioProject ID PRJNA419907).

134 **Quantification of bacteria and OsHV-1**

135 Quantification of OsHV-1, total bacteria 16S rDNA, and total *Vibrio* 16S rDNA was
136 performed using quantitative PCR (qPCR). All amplification reactions were analysed using a
137 Roche LightCycler 480 Real-Time thermocycler (qPHD-Montpellier GenomiX platform,
138 Montpellier University, France). A Labcyte Acoustic Automated Liquid Handling Platform
139 (ECHO) was used for pipetting into the 384-well plate (Roche). The total qPCR reaction
140 volume was 1.5 µl and consisted of 0.5 µl DNA (40 ng µl−1) and 1 µl LightCycler 480 SYBR
141 Green I Master mix (Roche) containing 0.5 µM PCR primer (Eurogenetec SA). Virus-specific
142 primer pairs targeted a DNA polymerase catalytic subunit (DP, ORF100, AY509253): Fw-
143 ATTGATGATGTGGATAATCTGTG and Rev-GGTAAATACCATTGGTCTTGTTCC
144 (Davison et al., 2005). Total bacteria specific primer pairs were the 341F-
145 CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC primers
146 targeting the variable V3V4 loops for bacterial communities (Klindworth et al., 2013). Total
147 *Vibrio* specific primer pairs were Fw-GGCGTAAAGCGCATGCAGGT and Rev-
148 GAAATTCTACCCCCCTCTACAG (Mansergh and Zehr, 2014). qPCR reactions were
149 performed with the following program: 95 °C for 10 min, followed by 40 cycles of

150 denaturation (95 °C, 10 s), hybridization (60 °C, 20 s) and elongation (72 °C, 25 s). After
151 these PCR cycles a melting temperature curve of the amplicon was generated to verify the
152 specificity of the amplification. Absolute quantification of OsHV-1 copies was calculated by
153 comparing the observed Cq values to a standard curve generated from the DNA polymerase
154 catalytic subunit amplification product cloned into the pCR4-TOPO vector. For total bacteria
155 and total *Vibrio* 16S rDNA, we used the relative quantification calculated by the $2^{-\Delta\Delta Cq}$
156 method (Pfaffl, 2001) with the mean of the measured threshold cycle values of two reference
157 genes (*Cg-BPI*, GenBank: AY165040, *Cg-BPI* F-ACGGTACAGAACGGATCTACG, *Cg-*
158 *BPI* R-AATCGTGGCTGACATCGTAGC and *Cg-actin*, GenBank: AF026063, *Cg-actin* F-
159 TCATTGCTCCACCTGAGAGG, *Cg-actin* R-AGCATTTCCTGTGGACAATGG).

160 **Sequence analyses**

161 The FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented into a galaxy
162 instance (http://sigenae-workbench.toulouse.inra.fr/galaxy/) was used to define Operational
163 Taxonomic Units (OTU), and computed taxonomic affiliations (Escudié et al., 2017). Briefly,
164 paired reads were merged using FLASH (Magoc and Salzberg, 2011). After denoising and
165 primer/adapters removal with cutadapt (Martin, 2011), *de novo* clustering was performed
166 using SWARM that uses a local clustering threshold, with aggregation distance d=3 (Mahé et
167 al., 2015). Chimera were removed using VSEARCH (*de novo* chimera detection) (Rognes et
168 al., 2016). Particularly, this method divided each sequence into four fragments, and then
169 looked for similarity with putative parents in the whole set of OTUs. We filtered the dataset
170 for singletons and we annotated OTUs using Blast+ against the Silva database (release 123,
171 September 2015) to produce an OTU and affiliation table in standard BIOM format.
172 Rarefaction curves of species richness were produced using the {phyloseq} R package, and
173 the ggrare function (McMurdie and Holmes, 2013). In order to compare samples for alpha and
174 beta diversity, we used the rarefy_even_depth function to subsample dataset to 5148 reads per
175 sample excluding chloroplasts. The alpha diversity metrics (Chao1 and Shannon) were
176 estimated at the OTU level with the estimate_richness function. Moreover, Pielou's measure
177 of species evenness was computed using the diversity function in {vegan}. We also used
178 phyloseq to obtain abundances at differents taxonomic ranks (for genus and family)
179 (tax_glom function). Because agglomerate of multi-affiliation and unknown taxa does not
180 make sense at higher taxonomic ranks, we only kept taxa having a true annotation for each
181 corresponding taxonomic rank. We computed Bray-Curtis dissimilarities to study beta
182 diversity, *i.e.*, distances between samples for OTU compositions (vegdist function, {vegan}).

183 **Statistical and multivariate analyses**

184 All statistical analyses were done using R v3.3.1 (R: a language and environment for
185 statistical computing, 2008; R Development Core Team, R Foundation for Statistical
186 Computing, Vienna, Austria [http://www.R-project.org]).

187 Principal coordinate analyses (pcoa, {vegan}) were computed to describe compositions of
188 microbial communities between samples using Bray-Curtis dissimilarities (vegdist, {vegan}).
189 Multivariate homogeneity of group dispersions was tested between microbial assemblages of
190 resistant and susceptible oyster families using 999 permutations (permutest, {vegan}). We
191 used DESeq2 (Love et al., 2014) (DESeq {DESeq2}) to identify OTUs having differential
192 abundances between resistant and susceptible oyster families. Heatmaps of significant
193 bacterial genera and families were computed using relative abundances and the heatmap.2
194 function ({gplots}).

195 We performed one-way ANOVA or non-parametric Kruskal-Wallis tests (when normality of
196 residuals was rejected (Shapiro test)) to compare alpha diversity metrics of microbiota. When
197 ANOVA or Kruskal–Wallis tests were significant, we then computed pairwise comparisons
198 between group levels (post-hoc analyses) using pairwise-t-tests or Dunn tests, respectively.

199 For all analyses, the threshold significance level was set at 0.05. P-values were corrected for
200 multiple comparisons using Benjamini and Hochberg's method (Benjamini and Hochberg,
201 1995) (p.adjust, {stats}).

202 **Results**

203 **Survival rates and disease development**

204 Full-sib progenies from one resistant (R_{F21}) and two susceptible (S_{F15} and S_{F32}) oyster families
205 were produced and reared in bio-secured conditions (Argenton hatchery, France). About 140
206 pathogen-free oysters per family were transferred to the infectious environment for five days
207 (Brest, Atlantic Ocean, temperature above 16°C) (Figure 1). Fourteen oysters per family were
208 then flash frozen for individual bacterial microbiota analyses. The other 126 oysters per
209 family were placed back in hatchery to monitor survival rates. As expected, all oysters from
210 the two susceptible families died, whereas all oysters from the resistant family survived
211 (Figure 1). This observation showed that oysters were contaminated during transplantation,
212 and then developed the disease. Quantification of OsHV-1 and bacteria at day 5 post-
213 transplantation showed that only three oysters from susceptible families displayed moderate
214 to important viral infection, and that only one individual displayed both high viral infection
215 and bacteraemia (Figure 2). These results suggested that five days of transplantation in the
216 field during an infectious period were long enough for oyster contamination, but not for
217 entering the pathogenesis process (except for three oysters).

218 The whole bacterial communities were sequenced using the 16S rRNA gene from 14 oysters
219 per family and per condition (except 13 oysters for S_{F32} in hatchery) (*i.e.*, 83 oyster-associated
220 microbiota for control and infectious conditions). In average, each sample contained 21423
221 sequences representing 1075 OTUs (Supplementary Figure 1, Supplementary Tables 1 and 2).
222 First, we searched for known opportunistic genera within microbiota of diseased oysters (de
223 Lorgeril et al., 2018). Nine out of ten previously identified genera were present in our dataset
224 (Figure 3). Most were abundant for the oyster highly infected by OsHV-1 and displaying
225 bacteraemia (SF15.S.R14) (Figures 2 and 3A). Both *Vibrio* and *Psychromonas* genera were
226 found within the three diseased oysters (Figure 3A). However, *Vibrio* occurred in the majority
227 of healthy individuals as well (Figure 3B).

228 **Microbiota assemblages before diseased development**

229 Although five days of transplantation were long enough for oyster contamination, only three
230 individuals entered the pathogenesis process as indicated by virus load. Thus, after discarding
231 these three individuals, it was possible to compare microbiota characteristics of the other
232 oysters between resistant and susceptible families before the onset of disease development.

233 First, we compared alpha diversity indices (Chao1, evenness and Shannon) between oyster
234 families (Figure 4 and Supplementary Table 3). This analysis showed that Chao1 increased
235 for all families between hatchery and the infectious environment. Moreover, evenness and
236 Shannon indices increased only for the resistant family R_{F21}. Notably, R_{F21} had higher
237 evenness than S_{F15} and S_{F32} during the infectious condition. Secondly, we computed a
238 principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarities to describe
239 microbiota compositions (Figure 5A). This ordination highlighted that oyster microbiota
240 changed between hatchery and the infectious environment for the three oyster families.
241 Unexpectedly, microbiota dispersion of R_{F21} was not lower than the susceptible families, and
242 even showed higher dispersions for the infectious condition than in hatchery (Figure 5B).

243 Altogether, these results suggested that microbiota assemblages highly changed between
244 hatchery and the infectious environment for the three families. Although we did not expect to
245 find higher microbiota dispersion for R_{F21}, our analyses revealed that the resistant family had
246 higher evenness than the susceptible families before the onset of disease development.

247 **Different bacteria between oyster families in hatchery and infectious environment**

248 Because resistant and susceptible oyster families had different microbiota characteristics
249 (evenness and dispersion) at the early steps of infection, we compared abundances of bacterial
250 taxa within hatchery to identify putative pathogens, opportunists or mutualists that were
251 consistently presents in microbiota before transplantation. Abundances were compared at two
252 taxonomic ranks, bacterial genera (Supplementary Table 4) and families (Supplementary
253 Table 5). Two genera (*Colwellia* and *Photobacterium*) (Supplementary Figure 2) and four
254 families (Anaplasmataceae, Colwelliaceae, Mycoplasmataceae, and Vibrionaceae) (Figure 6)
255 showed high abundances (>4% in at least one sample) and were significantly different
256 between resistant and susceptible oyster families.

257 We also compared bacterial taxa before disease development between resistant and
258 susceptible oyster families that were placed in the infectious environment. Except for
259 Mycoplasmataceae, significant bacterial taxa were not similar to hatchery-identified
260 candidates (Figure 7 and Supplementary Figure 3). Four genera (*Phormidium*, *Pseudomonas*,
261 *Thalassospira*, and *Umboniibacter*) (Supplementary Figure 3) and seven families
262 (Cyanobacteria (Subsection I, family I), Cyanobacteria (Subsection III, family I),
263 Mycoplasmataceae, Pseudomonadaceae, Rhodobacteraceae, Rhodospirillaceae, and

264 Spirochaetaceae) (Figure 7) had high abundances (>4% in at least one sample) and were
265 significantly different between resistant and susceptible oyster families.

266 **Discussion**

267 **Five days of transplantation allowed oyster contamination, but not disease development**

268 Oysters were transplanted for five days in the field during an infectious period. According to
269 previous observations (Dégremont, 2011; Petton et al., 2015), this time period was considered
270 sufficient for oyster contamination. We did not observed dead oysters when they were
271 sampled, but all individuals of susceptible families died eight days after they were placed
272 back to laboratory tanks. This observation showed that oysters were contaminated during
273 transplantation, and this exposure was sufficient for the further development of the complete
274 pathogenesis process.

275 High abundances of OsHV-1 and total bacteria were previously observed for a susceptible
276 family during disease development (de Lorgeril et al., 2018). In this study, most oysters
277 (except three) did not exhibit high abundances of OsHV-1 or bacteria after five days of
278 transplantation. As a consequence and after exclusion of these three oysters, we thus analysed
279 oyster microbiota before the onset of disease development (*i.e.,* at the early steps of infection).

280 **Microbiota evenness was linked to oyster resistance during transplantation**

281 According to a previous study (de Lorgeril et al., 2018), we expected to observe many
282 modifications of microbiota for susceptible oyster families during transplantation, but not or
283 few for the resistant one. In particular, we expected increase of alpha diversity indices (Chao1
284 and Shannon), and of microbiota dispersion for the susceptible families. Furthermore, we
285 hypothesized that the resistant family might have stable microbiota, because this characteristic
286 was linked to host homeostasis in many studies (Faith et al., 2013; Rungrassamee et al., 2016;
287 Zaneveld et al., 2017).

288 Unexpectedly, we found that alpha diversity indices and dispersion of microbiota highly
289 changed for the three oyster families (susceptible as well as resistant) when they were
290 transferred from hatchery to the infectious environment. Microbiota evenness was the only
291 index that discriminated resistant and susceptible families. It highlighted that OTU
292 abundances within resistant oysters were more equally distributed than within susceptible
293 oysters. This index may possibly have important effects on oyster homeostasis, because it was
294 already found to be positively linked to ecosystem productivity (Wilsey and Potvin, 2000;
295 Zhang et al., 2012), functional stability (Balvanera et al., 2005; Wittebolle et al., 2009), and
296 invasion resistance (Wilsey and Polley, 2002; De Roy et al., 2013).

297 **Putative opportunistic and/or pathogenic bacteria of susceptible oysters**

298 To date, most *C. gigas* diseases affecting juveniles in Europe were associated with
299 *herpesvirus* (OsHV-1 μVar) and *Vibrio* strains (Labreuche et al., 2006; Garcia et al., 2011;
300 Travers et al., 2014; Batista et al., 2015; Petton et al., 2015). Furthermore, other studies
301 highlighted the role of *Roseovarius crassostreae* (Boettcher, 2005; Maloy et al., 2007),
302 *Tenacibaculum soleae* (Burioli et al., 2017), and possibly Chlamydiales (Renault and
303 Cochennec, 1995) and *Arcobacter* sp. (Lokmer and Wegner, 2015; Burioli et al., 2017). Other
304 potential opportunistic bacteria were recently identified (de Lorgeril et al., 2018), such as
305 *Cryomorphaceae*, *Fusibacter*, *Marinobacterium*, *Marinomonas*, *Psychrilyobacter*,
306 *Psychrobium*, *Psychromonas*, and *Salinirepens*.

307 In this study, most of these genera were identified within the oyster that displayed high
308 abundances of OsHV-1 and bacteraemia. *Psychromonas* occurred within the three diseased
309 oysters, and might possibly be involved early in disease development, even if its pathogenic
310 role was not demonstrated so far. Furthermore, three bacterial families (Mycoplasmataceae,
311 Rhodospirillaceae, and Vibrionaceae) were linked to susceptible oysters in hatchery and/or
312 before disease development in the field. Among Vibrionaceae, *Photobacterium* genus was
313 mostly associated with S_{F32} in hatchery, and as well with low disease resistance oysters in a
314 previous study (King et al., 2019). Among Rhodospirillaceae, *Thalassospira* genus was
315 already identified in *C. gigas* oysters (Fernandez-Piquer et al., 2012). Even though
316 Rhodospirillaceae were found in diseased tissues of *Platygyra carnosus* corals (Ng et al.,
317 2015), there is no evidence that they may act as pathogens for oysters until now. Lastly,
318 Mycoplasmataceae family was abundant in *C. virginica* oysters from Hackberry Bay (King et
319 al., 2012), and decreased in oysters during experimental infections (de Lorgeril et al., 2018).
320 However, it was intriguing to find in this study that Mycoplasmataceae abundances were
321 significantly lower for resistant than susceptible oyster families in both hatchery and field
322 conditions, at the early steps of infection.

323 **Putative beneficial bacteria of resistant oysters**

324 Some observations suggested that bacteria might protect oysters from pathogens. For
325 example, *Aeromonas media* A199, *Phaeobacter gallaeciensis* and *Pseudoalteromonas* sp.
326 D41 improved survival of *C. gigas* against *Vibrio* strains when they were tested as probiotics
327 (Gibson et al., 1998; Kesarcodi-Watson et al., 2012). In particular, the *Pseudoalteromonas*
328 genus is known to produce a wide variety of biologically active secondary metabolites

329 (Kalinovskaya et al., 2004; Bowman, 2007). Finding beneficial bacteria could be interesting
330 and pave the way for prophylactic measures in oyster farming.

331 Here we identified three bacterial families associated with resistant oysters: Colwelliaceae,
332 Cyanobacteria (Subsection III, family I), and Rhodobacteraceae. Among Colwelliaceae, the
333 *Colwellia* genus was already found within *C. gigas* microbiota (Madigan et al., 2014). In this
334 study, this family and genus had high abundances for resistant oysters in hatchery, but they
335 were not significantly different between resistant and susceptible oyster families during
336 transplantation, suggesting a limited role in POMS. Moreover, although Rhodobacteraceae
337 was associated to juvenile oyster disease (Boettcher, 2005), this bacterial family was
338 commonly associated with oysters (Asmani et al., 2016; Takenaka et al., 2017; Arahal et al.,
339 2018). Strikingly, Cyanobacteria (Subsection III, family I) had low abundances in all
340 susceptible oyster microbiota before disease development, and were abundant in most
341 resistant oysters. Although cyanobacteria had a negative effect on other marine invertebrates
342 such as scleractinian corals (Meyer et al., 2016), they were already observed at high relative
343 abundances within oyster microbiota (Chauhan et al., 2014). Particularly, they were abundant
344 in the digestive gland, connective tissue, mantle, and gonad of oysters (Avila-Poveda et al.,
345 2014). Because cyanobacteria persisted in oyster tissues without signs of alterations, a
346 possible endosymbiotic relationship was even proposed (Avila-Poveda et al., 2014). Notably,
347 cyanobacteria were also negatively correlated to culturable *Vibrio* abundance in free-living
348 microbial communities (Turner et al., 2009). Hence, cyanobacteria might play a role of barrier
349 against pathogens from the genus *Vibrio*. In particular, photosynthetic activity of
350 cyanobacteria could inhibited pathogen growth through the production of reactive oxygen
351 species, such as observed for *Plasmodium* infection in *Anopheles gambiae* (Cirimotich et al.,
352 2011).

353 To conclude, we placed oysters from resistant and susceptible families in field condition for
354 five days during a period in favour of POMS. The analyses of oyster microbiota at the early
355 steps of infection revealed differences between resistant and susceptible oyster families.
356 These differences suggested that both the structure (evenness) and the composition (putative
357 opportunists, pathogens, mutualists) might predict oyster mortalities. Future studies should
358 test the role of Cyanobacteria (Subsection III, family I), Mycoplasmataceae,
359 Rhodobacteraceae, and Rhodospirillaceae within *C. gigas* microbiota. In particular, these
360 studies should evaluate the possibility of using Cyanobacteria (Subsection III, family I) as
361 probiotics for oyster farming.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets generated during the current study are available in the Sequence Read Archive repository under BioProject ID PRJNA419907 (to be released upon publication).

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Authors' contributions: CC, JDL, JME, YG, LD, GM and ET were involved in the study concept and design. BP was involved in the generation and maintaining of all animals used in this study. JDL, BP, JME, YG, GM and ET were involved in the collection of samples. CC, JDL, AL and ET were involved in data acquisition and analysis. CC and ET drafted the manuscript and all authors contributed to critical revisions and approved the final manuscript.

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600 **Legends of figures**

601 **Figure 1. Experimental design.** Three oyster full-sib families were produced in controlled
602 condition (hatchery), and placed for five days in the environment (Atlantic Ocean, latitude:
603 48.335263; longitude: -4.317922) during an infectious period (July 2016). Then, oysters were
604 flash frozen, DNA were extracted, and microbiota were sequenced using 16S rRNA gene-
605 metabarcoding. Oysters deployed in the field during five days were followed during 8 days in
606 the hatchery. Survival rates were recorded at endpoint.

607 **Figure 2. OsHV-1 and bacterial colonization in oysters at day 5 post-transplantation in**
608 **field condition during an infectious period. A.** The OsHV-1 load was quantified by qPCR
609 and expressed as viral genomic units per ng of oyster DNA. Relative quantification of total
610 bacteria (**B**), and total vibrio (**C**) abundance were measured by qPCR.

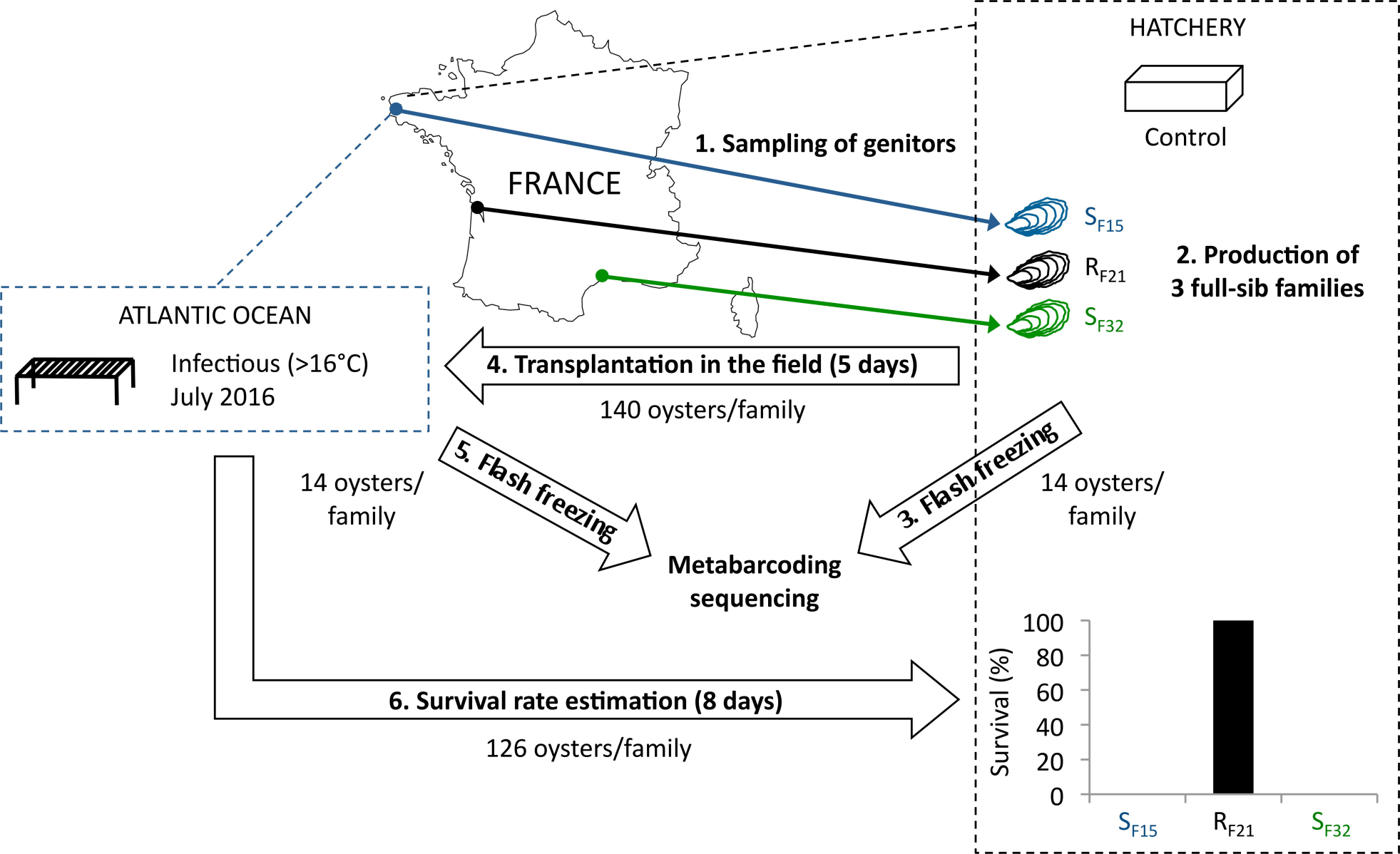
611 **Figure 3. Frequencies of known opportunistic bacterial genera associated with POMS.**
612 **A.** Positive oysters for OsHV-1 detection (SF15.I.R14, SF32.I.R08, and SF32.I.R14). **B.**
613 Negative oysters for OsHV-1 detection for each family. These opportunistic genera were
614 identified in a previous experimental infection (de Lorgeril et al., 2018). Frequencies above
615 and below 4% are displayed in red and blue, respectively.

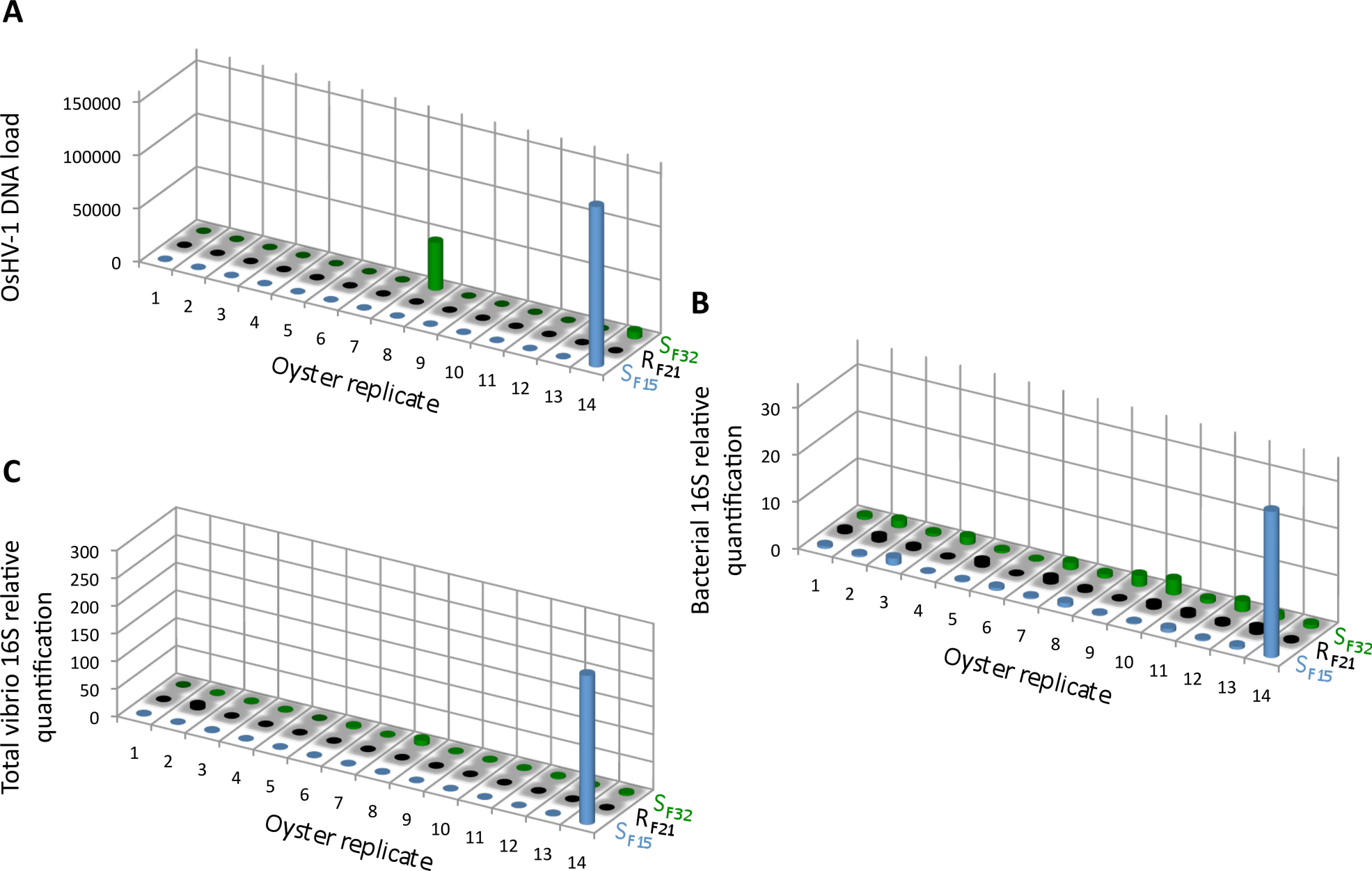
616 **Figure 4. Comparison of alpha diversity indices between oyster families in controlled**
617 **(hatchery) and infectious (field) conditions.** P-values correspond to global tests. Horizontal
618 lines above boxplots indicate significant pairwise comparisons between group levels (post-
619 hoc analyses).

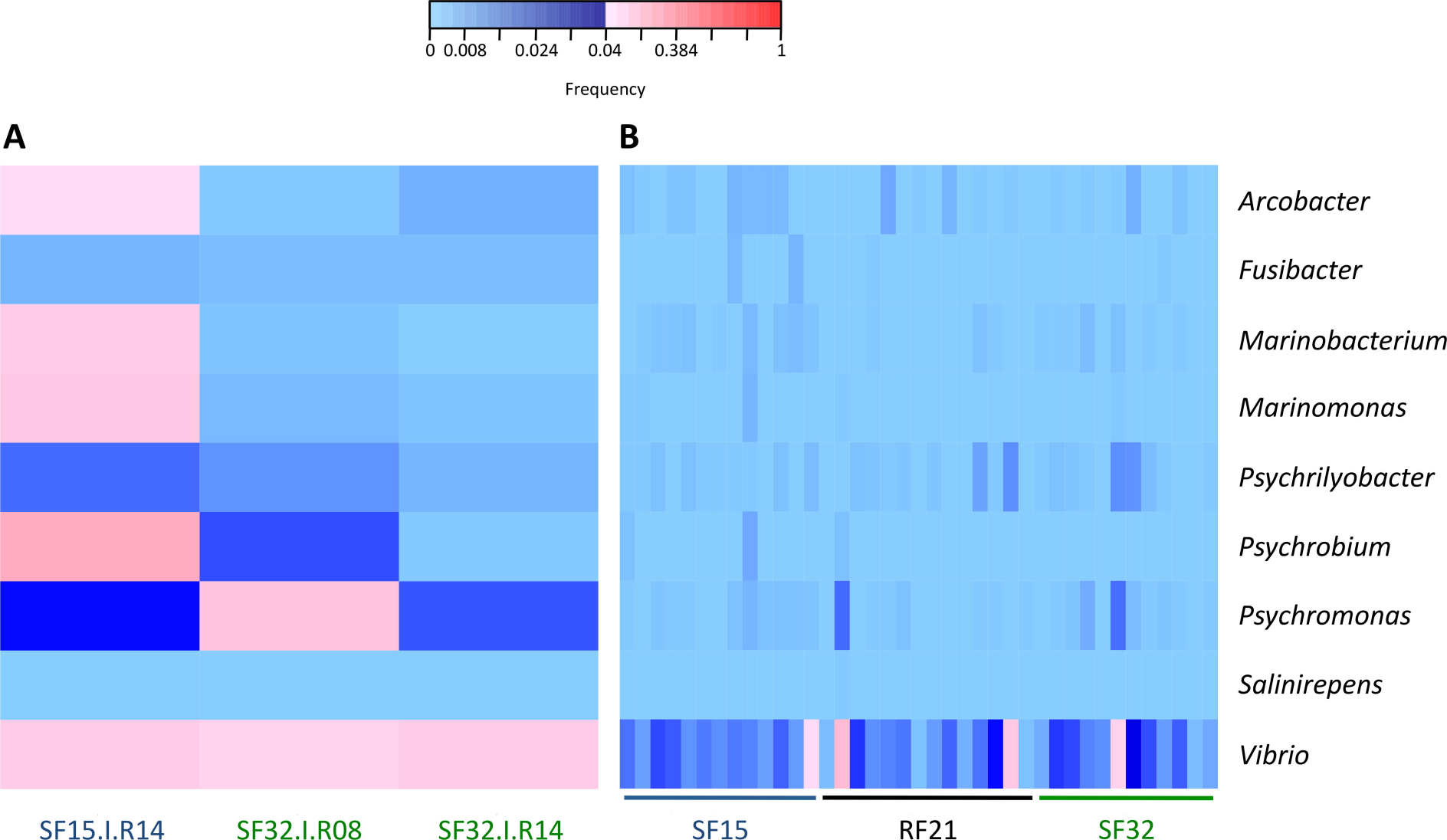
620 **Figure 5. Microbiota compositions between oyster families in controlled (hatchery) and**
621 **infectious (field) conditions. A.** Principal coordinate analysis (Bray-Curtis dissimilarities
622 between samples). **B.** Multivariate homogeneity of group dispersions. P-value corresponds to
623 the global test. Horizontal lines above boxplots indicate significant pairwise comparisons
624 between group levels (post-hoc analyses).

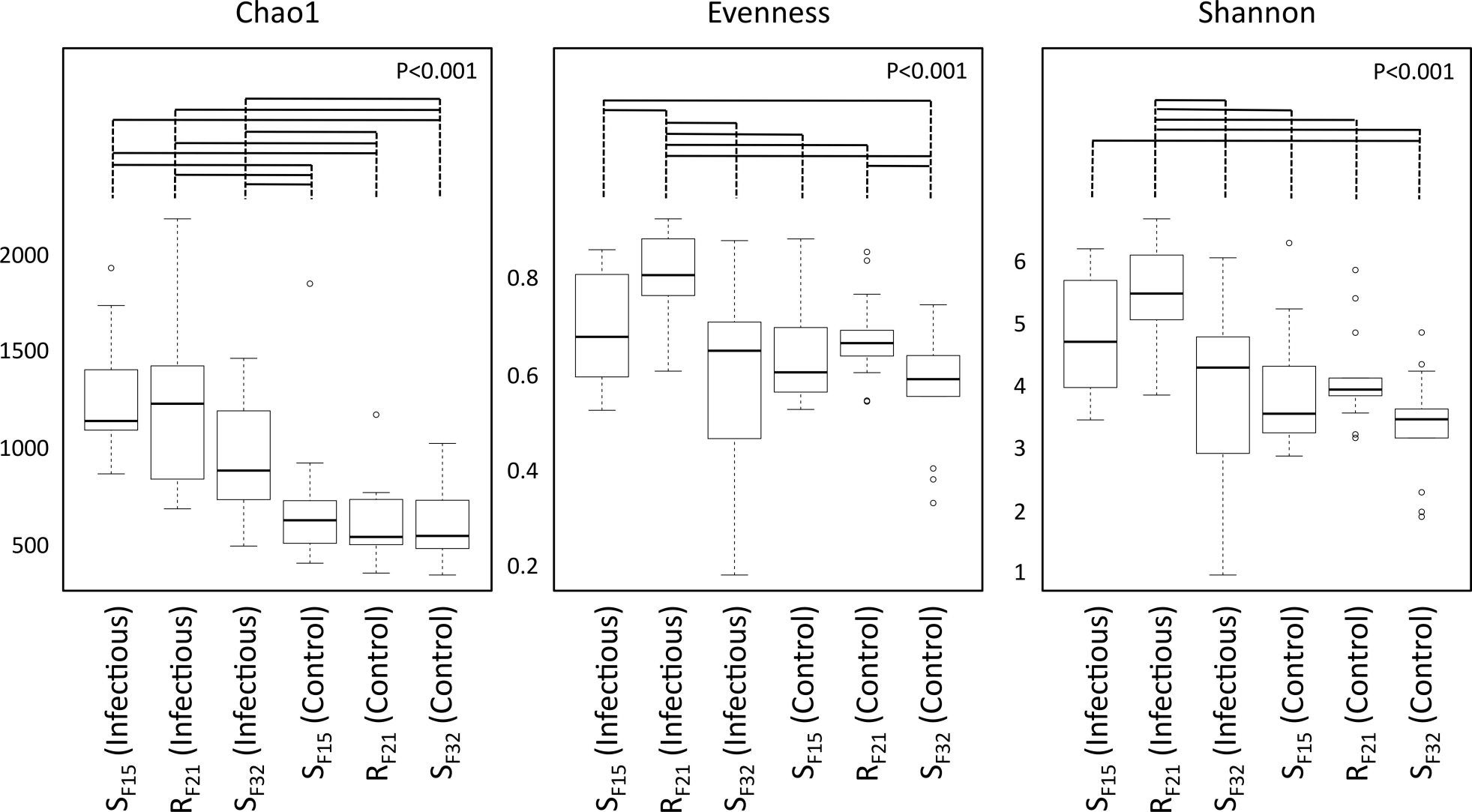
625 **Figure 6. Heatmap of bacterial families that were significantly different between**
626 **resistant and susceptible oyster families in the controlled condition (hatchery).** Only
627 bacterial families with a frequency above 4% in at least one sample are shown. Frequencies
628 above and below 4% are displayed in red and blue, respectively.

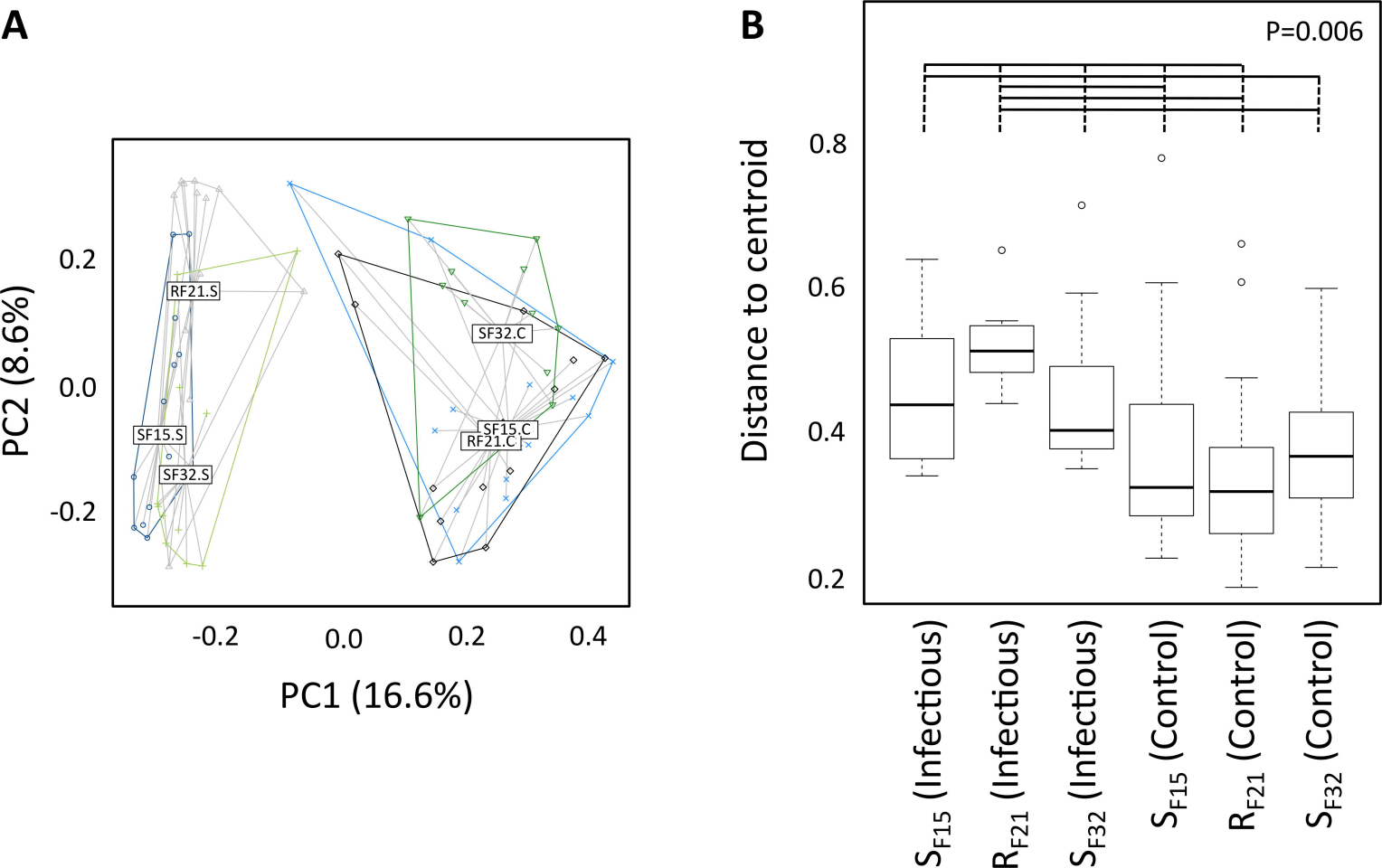
Figure 7. Heatmap of bacterial families that were significantly different between resistant and susceptible oyster families in the infectious condition (field). Only bacterial families with a frequency above 4% in at least one sample are shown. Frequencies above and below 4% are displayed in red and blue, respectively.

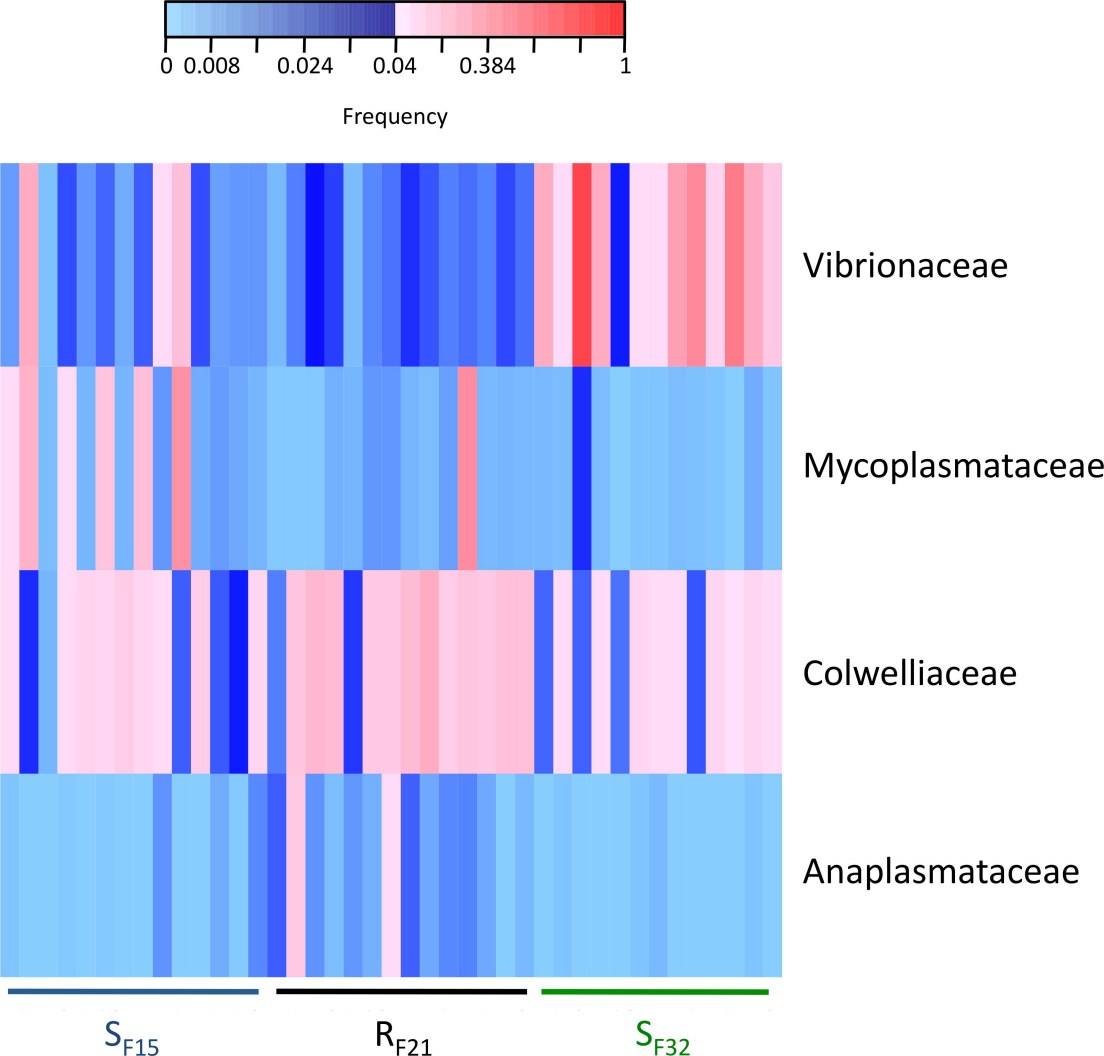

HATCHERY
Control
1. Sampling of genitors
FRANCE
S_{F15}
R_{F21}
S_{F32}
2. Production of 3 full-sib families
ATLANTIC OCEAN
Infectious (>16°C)
July 2016
4. Transplantation in the field (5 days)
140 oysters/family
5. Flash freezing
3. Flash freezing
14 oysters/ family
14 oysters/ family
Metabarcoding sequencing
6. Survival rate estimation (8 days)
126 oysters/family
Survival (%)
100
80
60
40
20
0
S_{F15}
R_{F21}
S_{F32}

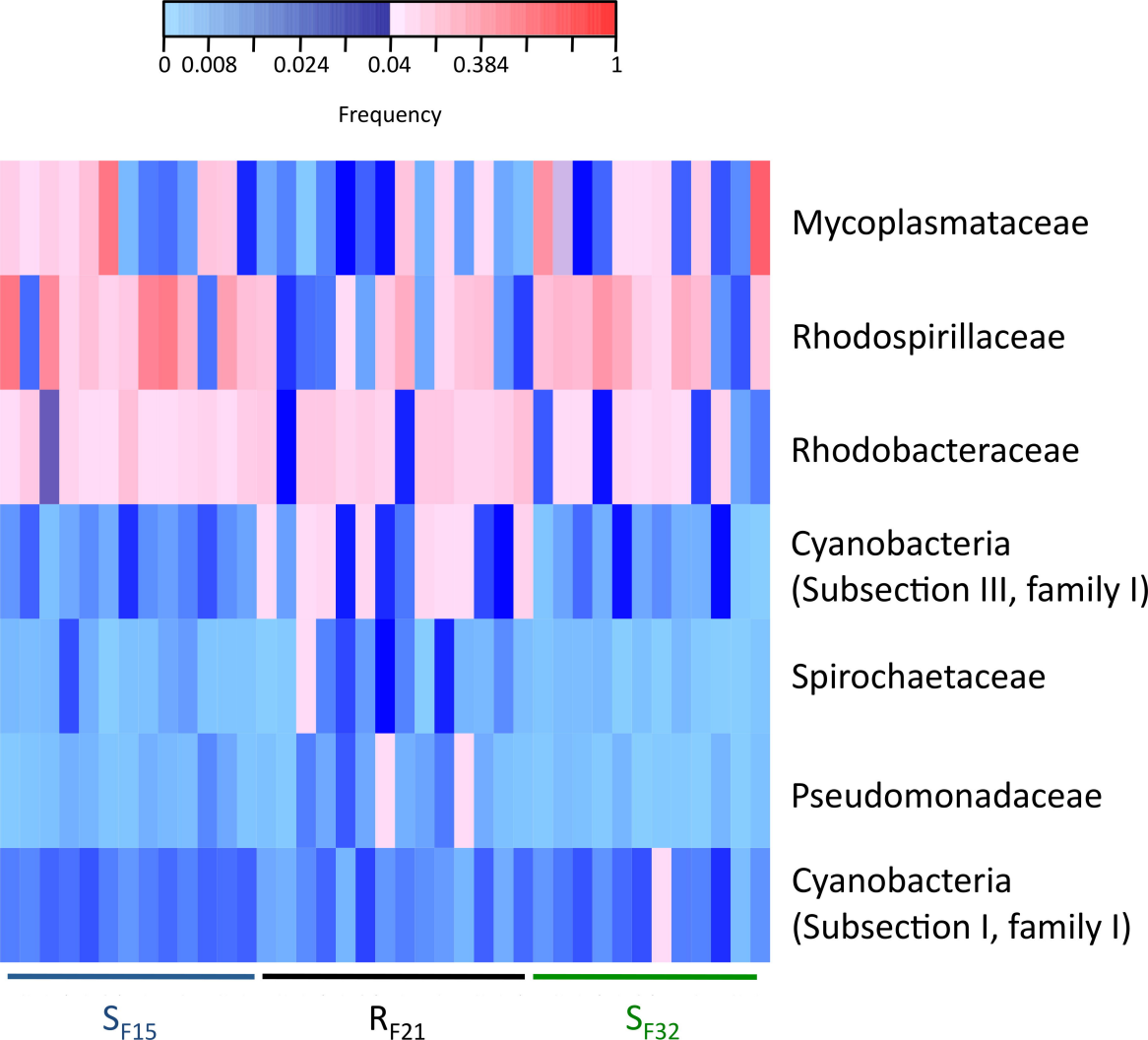

A
OsHV-1 DNA load
150000
100000
50000
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Oyster replicate
S_{F32}
R_{F21}
S_{F15}
B
Bacterial 16S relative quantification
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Oyster replicate
S_{F32}
R_{F21}
S_{F15}
C
Total vibrio 16S relative quantification
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Oyster replicate
S_{F32}
R_{F21}
S_{F15}


0
0.008
0.024
0.04
0.384
1
Frequency
A
B
Arcobacter
Fusibacter
Marinobacterium
Marinomonas
Psychrilyobacter
Psychrobium
Psychromonas
Salinirepens
Vibrio
SF15.I.R14
SF32.I.R08
SF32.I.R14
SF15
RF21
SF32


Chao1
P<0.001
2000
1500
1000
500
Evenness
P<0.001
0.8
0.6
0.4
0.2
Shannon
P<0.001
6
5
4
3
2
1
S_{F15} (Infectious)
R_{F21} (Infectious)
S_{F32} (Infectious)
S_{F15} (Control)
R_{F21} (Control)
S_{F32} (Control)


A
RF21.S
SF15.S
SF32.S
SF32.C
SF15.C
RF21.C
0.2
0.0
-0.2
PC2 (8.6%)
-0.2
0.0
0.2
0.4
PC1 (16.6%)
B
P=0.006
Distance to centroid
0.8
0.6
0.4
0.2
S_{F15} (Infectious)
R_{F21} (Infectious)
S_{F32} (Infectious)
S_{F15} (Control)
R_{F21} (Control)
S_{F32} (Control)


0
0.008
0.024
0.04
0.384
1
Frequency
Vibrionaceae
Mycoplasmataceae
Colwelliaceae
Anaplasmataceae
S_{F15}
R_{F21}
S_{F32}


0
0.008
0.024
0.04
0.384
1
Frequency
Mycoplasmataceae
Rhodospirillaceae
Rhodobacteraceae
Cyanobacteria
(Subsection III, family I)
Spirochaetaceae
Pseudomonadaceae
Cyanobacteria
(Subsection I, family I)
S_{F15}
R_{F21}
S_{F32}