1 **Bacteria with antibacterial activities isolated from *Magallana gigas* microbiota as**
2 **potential probiotics against *Vibrio aestuarianus* infections in oyster farming**

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22 ## Abstract

23 Introduction: Oyster farming is a significant industry worldwide, but it is threatened by various
24 diseases such as Pacific Oyster Mortality Syndrome or vibriosis. *V. aestuarianus* is a major
25 cause of mortality for market-size oysters, resulting in significant economic losses for oyster
26 farmers. Among the various control methods developed, probiotics appear to be a promising
27 approach. More specifically, the use of the antibacterial activity of bacteria from the natural
28 microbiota of the oyster *Magallana gigas* appears to be a sustainable solution against *V.*
29 *aestuarianus* infections.

30 Results: Our study investigated the probiotic potential of bacteria isolated from the microbiota
31 of *M. gigas* oysters. We screened a collection of 334 bacteria against eight target pathogens,
32 including *V. aestuarianus*, and identified 78 bacteria with antibacterial activity for which eight
33 retained this activity in their culture supernatants. Five strains were selected for further testing
34 and exposed to oysters prior to *V. aestuarianus* infection. Our results show that four strains
35 significantly reduced oyster mortality, with a maximum reduction of 70%. In addition, changes
36 in oyster microbiota composition were observed following exposure, but the administered
37 bacteria were not detected in the microbiota.

38 Conclusion: Our findings demonstrate the potential of oyster microbiota-derived bacteria as
39 probiotics for disease control in oyster farming. This approach could provide a sustainable and
40 environmentally friendly solution for the oyster farming industry. Further research is needed to
41 understand the underlying mechanisms and to develop effective probiotic-based strategies for
42 preventing *V. aestuarianus* infection.

43

44 Key words:

45 *Magallana gigas*; Antibacterial activities; Microbiota; *Vibrio aestuarianus,* Aquaculture

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48 **Introduction:**

49 The Pacific Oyster *Magallana gigas* (formerly known as *Crassostrea gigas*), is the most widely
50 cultivated oyster species in the world, contributing significantly to the aquaculture industry
51 (Food and Agriculture Organisation 2022). Nevertheless, the farming of *M. gigas* encounters
52 substantial difficulties due to recurrent infectious diseases, leading to high annual mortality
53 rates (Friedman et al. 2005; Cotter et al. 2010; Pernet et al. 2012; Azéma et al. 2015). Since
54 2001, mass mortality of adult *M. gigas* has been reported in France, in association with the
55 detection of the bacterium *Vibrio aestuarianus* (Garnier et al. 2008). This bacterium is a harmful
56 primary pathogen with chronic mortality reaching a cumulative mortality rate up to 30%. This
57 represents important economic consequences since *V. aestuarianus* preferentially infects
58 market size oysters which have been raised for several years (Azéma et al. 2017; Lupo et al.
59 2019). Other *Vibrio* species have been associated with mortality episodes affecting *M. gigas*
60 oysters at different stages of development. Notably, *V. coralliilyticus* has been linked to massive
61 mortalities of *M. gigas* larvae (Richards et al. 2015; Travers et al. 2015; Ushijima et al. 2022).
62 Spat / juveniles are affected by *V. crassostreae* (Dégremont et al. 2021; Cowan et al. 2023) and
63 *V. harveyi* (Dégremont et al. 2021; Oyanedel et al. 2023).

64 Efforts to address the challenges of *Vibrio* infections affecting *M. gigas*, have led to various
65 strategies build upon growing knowledge on oysters. One promising method involves the use
66 of genetic selection to breed pathogen-resistant oysters (Dégremont et al., 2015, 2020), although
67 this approach has limitations, such as the potential selection of trade-offs that could negatively
68 impact the commercial value of *M. gigas*. Furthermore, the discovery of immune priming in *M.*
69 *gigas* has paved the way for innovative applications using heat-killed *V. splendidus* (Zhang et
70 al. 2014), which provide protection against *V. splendidus* infections. Research on disease
71 prevention in molluscs based on the use of probiotics has been ongoing for decades (Yeh et al.
72 2020; Takyi et al. 2023, 2024; Dantan et al. 2024a, b; Muñoz-Cerro et al. 2024). Probiotics
73 display their positive benefits through a variety of methods, including direct pathogen inhibition
74 via competition for nutriments or production of antimicrobial compounds, but also indirect
75 immunomodulatory effects (Lazado and Caipang 2014; Yan et al. 2014; Peixoto et al. 2017;
76 Khademzade et al. 2020). Previous studies have demonstrated that bacterial strains
77 *Pseudoalteromonas sp.* hCg-6 and *Pseudoalteromonas sp.* hCg-42 isolated from *M. gigas*
78 haemolymph, displayed *in vitro* antibacterial activity against marine pathogens such as *V.*
79 *splendidus*, *V. tapetis*, *V. harveyi* and *Aeromonas salmonicida* (Defer et al. 2013; Desriac et al.
80 2014; Offret et al. 2018). Furthermore, exposure to *Pseudoalteromonas* sp. hCg-6 has been

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81 shown to enhance the survival of *Haliotis tuberculata* abalone during infection with *V. harveyi* 82 ORM4 (Offret et al. 2018). In addition, antimicrobial-producing bacteria have been employed 83 as a strategy to improve the survival of oysters against bacterial infections. For instance, 84 *Crassostrea virginica* larvae exposed to *Bacillus pumilus* RI06-95 exhibited significantly 85 increased survival rates during challenge with *Vibrio coralliilyticus* (Sohn et al. 2016). 86 Similarly, an exposure of *M. gigas* larvae to *Pseudoalteromonas sp.* was found to inhibit the 87 growth of *Vibrio coralliilyticus*, thereby improving larval survival during subsequent infection 88 (Madison et al. 2022).

89 In this article, we investigated the potential of bacteria isolated from the natural microbiota of 90 *M. gigas* to protect oysters against *V. aestuarianus* infection. For this, we firstly screened a 91 collection of bacteria previously isolated from *M. gigas* associated microbiota (Dantan et al. 92 2024b) for their antibacterial activity *in vitro* against four oyster pathogenic Vibrio. sp. (Travers 93 et al. 2015) and against opportunistic bacteria associated with the POMS disease (de Lorgeril 94 et al. 2018; Clerissi et al. 2022). Secondly, selected candidate bacteria were tested for their 95 effect against *V. aestuarianus* infection, and we investigated the impact of the administered 96 bacteria on the microbiota of the exposed oysters.

97

98 Materials and methods:

99

100 Screening for antibacterial activities of bacteria isolated from *M. gigas* microbiota

101 Eight bacterial strains were selected as targets for the screening of antibacterial activities. Four 102 of them were pathogenic *Vibrio* for oysters at different developmental stages: *Vibrio* 103 *aestuarianus* 02/041 (Garnier et al. 2008), *Vibrio coralliilyticus* 06/210 (Dégremont et al. 104 2021), *Vibrio crassostreae* J2-9 (Lemire et al. 2015) and *Vibrio harveyi* Th15_O_A01 105 (Oyanedel et al. 2023). The four others are bacteria associated with POMS dysbiosis according 106 to (de Lorgeril et al. 2018; Clerissi et al. 2022): *Amphitrea sp.* 14/114-3T2, *Marinobacterium* 107 *sp.* 05/091-3T1, *Marinomonas sp.* 12/107-2T2, *Pseudoalteromonas sp.* 09/041-1T3 108 (**Supplementary_File_1 Table S1**). Target bacteria were provided by the French National 109 Reference Laboratory (Ifremer, La Tremblade, France) or came from previous projects carried 110 out in our laboratory (Oyanedel et al. 2023).

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111 All strains were cultivated from glycerol stock in 10 mL Marine Broth (MB) at 20°C for 48
112 hours under constant agitation (100 rpm), then the OD_{600} was determined using BioPhotometer
113 (Eppendorf). The cultures were diluted into fresh MB to a final concentration of 10^6 CFU/mL
114 prior to inoculation of Marine Agar plates by inundation. The 334 bacteria from our previously
115 described bacterial collection isolated from *M. gigas* microbiota (**Supplemenary_File_1 Table**
116 **S2**) (Dantan et al. 2024b) were then tested for their antibacterial activity against each of the
117 target. Each bacteria from the collection were grown from glycerol stock in 2 mL MB at 20°C
118 under constant agitation (100 rpm) for 48h before being distributed onto 4 different 96 well
119 microplates. These microplates were then duplicate using a microplate pin replicator on new 96
120 well microplate containing fresh MB and incubated overnight at 20°C on MB media under
121 constant agitation (100 rpm) and then deposited in arrays of 8x12 (2 µL) spots using a
122 microplate pin replicator on marine agar plate previously inoculated with the target bacteria. A
123 2 µL spot of kanamycin (50 µg/mL) was used as positive control and a 2 µL spot of sterile
124 Marine Broth as a negative control. Marine agar plates without target bacteria were used as
125 growth and purity control of the bacteria from collection. Agar plates were then incubated at
126 20°C for two days and were then photographed using Gel Doc XR (Biorad, CA, USA) and the
127 "Flamingo" filter to visualise a potential halo of inhibition characteristic of antibacterial
128 activity. For supernatant assay, the same target bacteria were used. Prior to the test, the bacteria
129 were cultured in Marine Broth media on 96 well plates during 72h at 20°C. After the incubation
130 period, the 96 well plate was centrifugated during 10 minutes at 4000 rpm. The supernatants
131 were then carefully transferred into new 96 well plates and heated at 100°C for 5 minutes to
132 kill the possible remaining bacteria. Then, 2 µL spots were deposited on the marine agar plates
133 previously flooded with the target bacteria as describe above.

134

135 **Oysters used as donors and recipient**

136 Oysters were produced at the Ifremer hatchery in La Tremblade in February 2021. Briefly, 25
137 females and 25 males were used to produce100 bi-parental families (each male was mated to
138 four females, and each female was mated to four males).

139 Each family was raised in separated tank during the larval stage, and then each family was
140 settled in separate trays until two-months old. Then, 150 spat of each family were individually
141 counted and mixed together to produce a batch. This batch of mixed families was transferred to
142 the Ifremer nursery in Bouin in May 2021 until the experimental infection in November 2021.

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143 All oysters were kept in our controlled facilities using UV-treated seawater until their
144 evaluations. Animals were fed *ad libitum* using a cultured phytoplankton diet (*Isochrysis*
145 *galbana*, *Tetraselmis suecica* and *Skeletonema costatum*).

146

147 **Oyster exposure to bacteria selected for a potential beneficial effect**

148 Recipient oysters were distributed between seven 40 L tanks filled with UV-treated seawater
149 and maintained at 20°C with adequate aeration. Each tank contained 75 adult oysters (mean
150 individual weight = 29.68 ± 8.03 g). These recipient oysters were either exposed to one of the
151 five bacterial strains selected for their antibacterial activities, one control bacteriocin producing
152 strain (*Pseudoalteromonas sp.* hCg-42) which has been previously isolated from *M. gigas*
153 haemolymph (Defer et al. 2013) or to sterile artificial seawater (control).

154 Prior to exposure, the bacteria (*Pseudoalteromonas* sp. hCg-42, *Bacillus* sp. ARG61,
155 *Halomonas* sp. LTB66, *Cytobacillus* sp. ARC29, *Yoonia* asp. THAU59 and *Vibrio* sp. LTB1)
156 were individually cultured in 10 mL of MB media for 48h at 20°C under constant agitation and
157 then, 1 mL of each bacterial culture was inoculated into 10 mL fresh MB media and incubated
158 at 20°C under constant agitation. After 48 hours of incubation, the OD_{600} was measured, and
159 the appropriate amount of bacteria (1 OD_{600} unit = 8×10^8 CFU/mL) was collected, centrifuged
160 at 4000 rpm for 2 minutes and the supernatant was discarded. The pellets were then resuspended
161 in 10 mL sterile artificial seawater and added immediately to tanks containing the adult oysters
162 so that the final concentration in the tank was adjusted to 10^4 CFU/mL. The selected bacteria
163 were added individually during seven days and were renewed two times without water renewal
164 at days two and four (**Figure 1**).

165 Five oysters exposed to each condition were sampled at day seven (D7) of the bacterial exposure
166 to perform molecular analysis. Sampled oysters were grounded in liquid nitrogen (Retsch
167 MM400 mill) to a powder that was stored at -80°C until subsequent DNA extraction.

168

169 ***Vibrio aestuarianus* experimental infection by cohabitation**

170 A *V. aestuarianus* experimental infection was performed immediately following the bacterial
171 exposure to recipient oysters. A cohabitation protocol was used as previously described in (De
172 Decker and Saulnier 2011). The *V. aestuarianus* 02/041 was grown in Zobell medium at 22°C
173 for 24h under agitation. The bacterial concentration was determined by measuring the OD_{600}

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174 and was adjusted to OD_{600} of 1 representing 5.10^8 bacteria per mL in artificial seawater. Seventy
175 donor oysters were injected in the adductor muscle with 100µL of the *V. aestuarianus* 02/041
176 suspension and were then equally distributed among the five tanks (**Figure 1**). The donors were
177 from the same oyster population as the recipient oysters exposed to the strains showing
178 antibacterial activities. A ratio 1:1 was used for donor and recipient oysters (Azéma et al. 2017).
179 After 48 hours of cohabitation and before first mortality, donors were removed from the tanks.
180 The mortality of recipient oysters exposed to selected bacteria and control oysters, was then
181 recorded during 17 days by recording the dead oyster every day, and all the dead oysters were
182 removed from the tanks.

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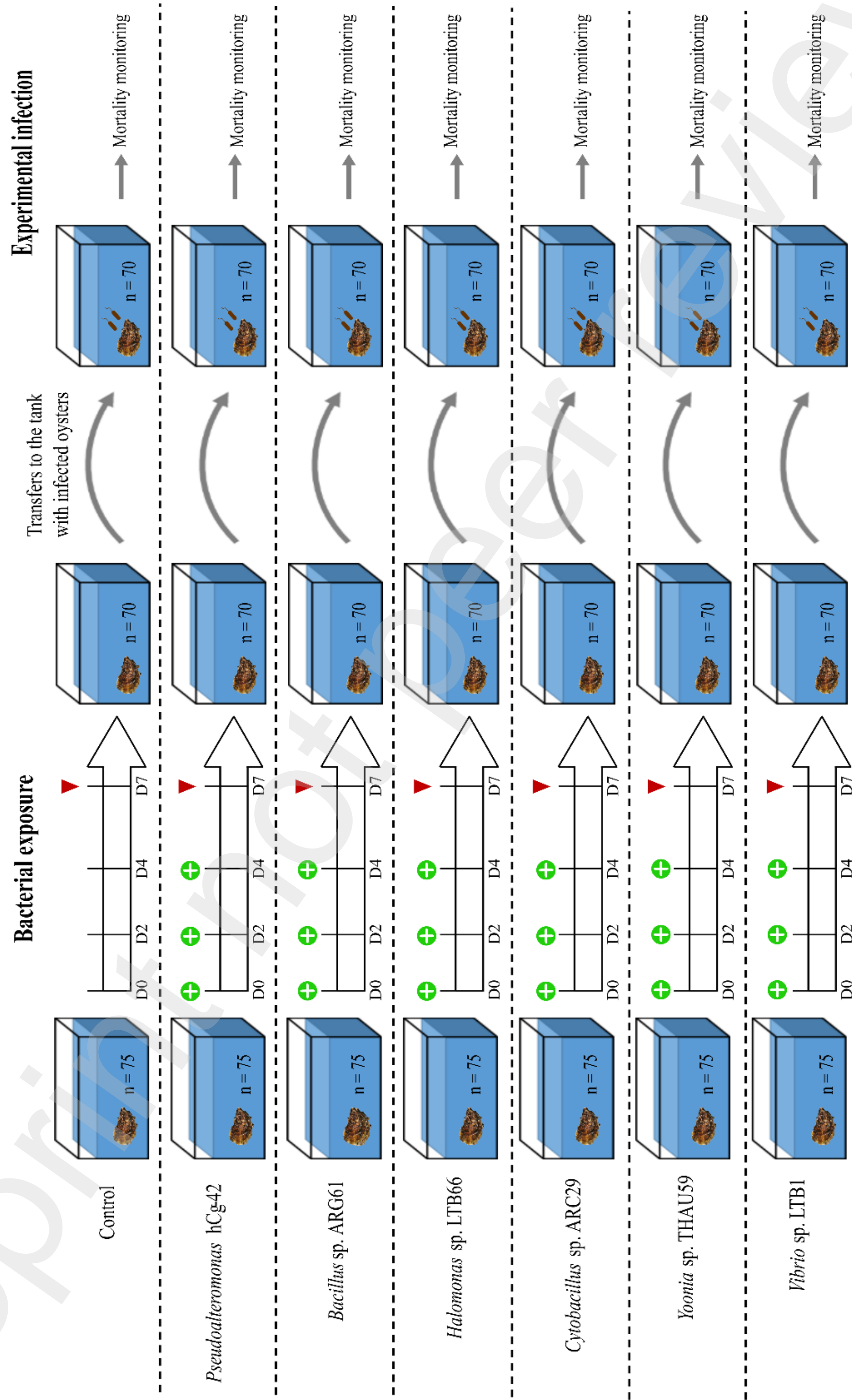

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184 **Figure 1: Overall experimental design for bacterial exposure and experimental infections**
185 **performed with the NTA oyster population.**
186 Recipient oysters (n=75) were placed in 40L tank filed with UV-treated seawater and
187 maintained at 20°C with adequate aeration. Oysters were then exposed to individual selected
188 bacterial strains during seven days with a renewal every two days (indicated by the "+" sign in
189 the green circle). At the end of bacterial exposure, 5 oysters per tank were sampled, flash-frozen
190 into liquid nitrogen and stored at -80°C for molecular analysis (indicated by red triangles). Right
191 after the bacterial exposure, remaining recipient oysters were transferred into 5 new tanks
192 containing donor oysters injected with *V. aestuarianus* in order to realise an experimental
193 infection.

194

195 **Statistical Analysis of oyster mortality**

196 Oyster mortality was analysed using survival analysis performed on R (v 4.2.1) (R Core Team
197 2022) with the package survminer (v 0.4.9) (https://cran.r-
198 project.org/web/packages/survminer/index.html). The Kaplan-Meier method was used to
199 represent the cumulative survival rate. A log-rank test was used to determine the difference
200 between the conditions and post-hoc pairwise comparisons with Bonferroni corrected p-value
201 were used to define which values were significantly different from the control. A multivariate
202 Cox proportional hazards regression model was used to compute Hazard-Ratio (HR) with
203 confidence intervals of 95%.

204

205 **Bacteria and oyster DNA extraction**

206 DNA extraction from oysters collected during bacterial exposure was performed from frozen
207 powders using DNA from the tissue Macherey-Nagel kit according to the manufacturer's
208 protocol. Prior to 90 min of enzymatic lysis in the presence of proteinase K, an additional 12-
209 min mechanical lysis (Retsch MM400 mill) was performed with zirconia/silica beads
210 (BioSpec). DNA concentrations were checked with a Qubit® 2.0 Fluorometer (Thermo
211 Scientific) and adjusted when necessary.

212 Bacterial DNA for the candidate strains used to constitute the mock community was extracted
213 as described in (Dantan et al. 2024b)

214

215 **16S rDNA library construction and sequencing**

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216 Library construction (with primers 341F 5'-CCTAYGGGRBGCASCAG and 806R 5'-
217 GGACTACNNGGGTATCTAAT targeting the 16S V3V4 region) and sequencing on a MiSeq
218 v2 (2x250 bp) were performed by ADNid (France).

219

220 **Bioinformatic pipeline for 16S barcoding analysis**

221 Sequencing data obtained in this study were processed with the SAMBA (v 3.0.2) workflow
222 developed by the SeBiMER (Ifremer's Bioinformatics Core Facility). Briefly, Amplicon
223 Sequence Variants (ASV) are constructed with DADA2 (Callahan et al. 2016) and the QIIME2
224 dbOTU3 (v 2020.2) tools (Bolyen et al. 2019), Due to the known diversity overestimation
225 generated by DADA2, an additional step of ASV clustering has been performed using dbOTU3
226 algorithm (Olesen et al. 2017) and contaminations were removed with microDecon (v 1.0.2)
227 (McKnight et al. 2019). Taxonomic assignment of ASVs was performed using a Bayesian
228 classifier trained with the Silva database v.138 using the QIIME feature classifier (Wang et al.
229 2007). Finally, community analysis and statistics were performed on R (R version 4.2.1) (R
230 Core Team 2022) using the packages phyloseq (v 1.40.0) (McMurdie and Holmes 2013), Vegan
231 (v 2.6-4) (Oksanen et al. 2022) and MicroEco (v. 1.9.1) (Liu et al. 2021).

232 For beta-diversity, the ASVs counts were preliminary normalized with the "rarefy_even_depth"
233 function (rngseed = 711) from the package phyloseq (v 1.40.0)(McMurdie and Holmes 2013).
234 Principal Coordinates Analysis (PCoA) were computed to represent dissimilarities between the
235 samples using the Bray-Curtis distance matrix. Differences between groups were assessed by
236 statistical analyses (Permutational Multivariate Analysis of Variance) using the adonis2
237 function implemented in the vegan package (2.6-4) (Oksanen et al. 2022).

238

239 **Detection of administered bacteria in 16S barcoding dataset**

240 In order to search for the specific presence of the administered bacteria, we first produced full-
241 length 16S DNA for the identification of the selected candidate bacteria (Dantan et al. 2024b).
242 In parallel, a mock community composed of equal amounts of DNA from four of the
243 administered bacteria (*Bacillus* sp. ARG61, *Vibrio* sp. LTB1, *Halomonas* sp. LTB66, and
244 *Cytobacillus* sp. ARC29) was also submitted to 16S amplicon sequencing in order to validate
245 our method. We then aligned 16S reference sequences of the administered bacteria against all
246 the ASV sequences from the dataset using BLAST (Altschul et al. 1990). We considered ASVs

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247 sequences with a percentage of identity superior to 99% along the full V3V4 marker sequence
248 as being our administered bacteria.

249

250 ## Results:

251

252 **78 bacterial strains displayed an antibacterial activity against**

253 We previously isolated 334 bacteria from the microbiota of *M. gigas* oysters from four different
254 geographical sites (Brest Bay, La Tremblade in Marennes-Oleron Bay, Arcachon Bay and Thau
255 Lagoon) (Dantan et al. 2024b). This collection was screened for their antibacterial activity
256 against eight target bacteria (**Supplementarry_File_1_Table S1**). Among the 334 bacteria
257 from the collection, 78 strains showed an inhibition area around bacterial colony. Among these
258 strains, 32 showed antibacterial activity against Vibrios (17 against *V. harveyi* Th15_O_A01,
259 14 against *V. aestuarianus* 02/041, 12 against *V. coralliilyticus* 06/210 and 8 against *V.*
260 *crassostreae* J2-9) and 65 against opportunistic bacteria associated with POMS disease (49
261 against *Marinomonas sp.* 12/107-2T2, 8 against *Pseudoalteromonas sp.* 09/041-1T3, 6 against
262 *Amphitrea sp.* 14/114-3T2, and 2 against *Marinobacterium sp.* 05-091-3T1) (**Figure 2A**).

263 The 76 bacteria presenting an antibacterial activity by co-culture were further screened for the
264 antibacterial activity of their culture supernatant. Thus, height bacteria conserved their activity
265 in the culture supernatant. Among them, three bacterial strains (*Halomonas sp.* LTB66,
266 *Halomonas sp.* LTB97 and *Vibrio sp.* LTB1) presented antibacterial activities in their
267 supernatant against six of the eight target bacteria, two strains (*Bacillus sp.* ARG61 and
268 *Cytobacillus sp.* ARC29) presented antibacterial activities in their culture supernatant against
269 two of the height target bacteria and the three other strains had antibacterial activities in their
270 supernatant against one of the eight target bacteria. (**Figure 2B**).

271 Based on these results, we selected five bacterial strains for further assay. The bacteria were
272 chosen to ensure representation from each geographic origin in the selected bacteria, preferably
273 with antibacterial activity present in the supernatant culture. Thus, *Bacillus sp.* ARG61 from
274 Brest Bay, *Halomonas sp.* LTB66 and *Vibrio sp.* LTB1 from La Tremblade in Marennes-Oleron
275 Bay, *Cytobacillus sp.* ARC29 from Arcachon Bay and *Yoonia sp.* THAU59 from Thau Lagoon
276 were selected for protection assay during an experimental infection.

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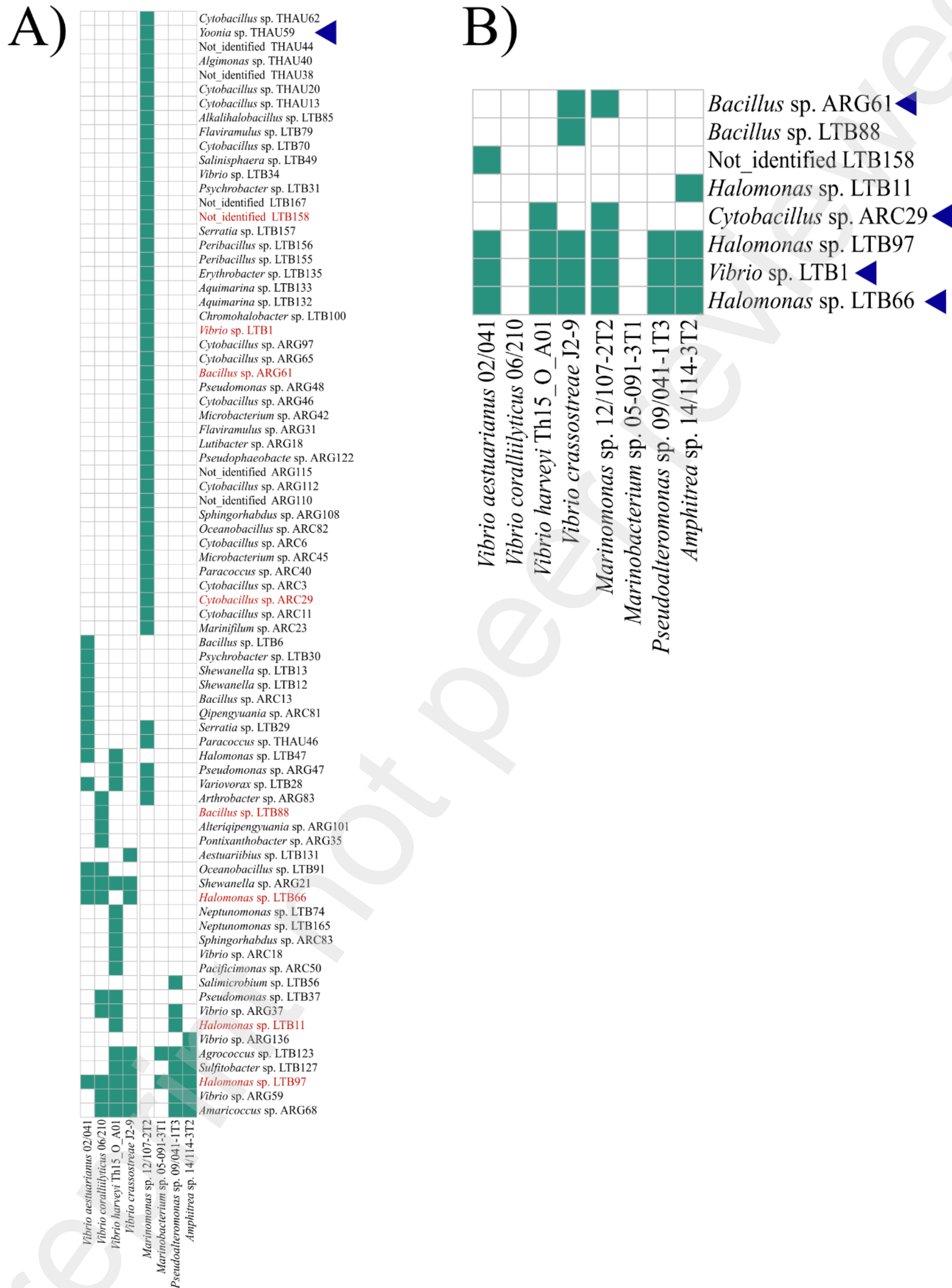

278

279 **Figure 2: Antibacterial activities of bacteria isolated from oysters against the eight**
280 **target bacteria.**
281 The plot represents all the bacteria of the collection having an antibacterial activity against the
282 different target bacteria (A) by coculture and (B) in the culture supernatant. Positive

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283 antibacterial activity tests are represented by green tiles. Bacteria written in red (A) are those
284 for which activity was found in the culture supernatants (B). Bacteria selected for
285 experimental infection assay are indicated by a blue triangle.

286

287 **Four bacterial strains induced a significant reduction of mortality risk during *V.***
288 ***aestuarianus* infection.**

289 To test if exposure to selected bacteria with an antibacterial activity can induce a protective
290 effect against *V. aestuarianus* infection, adult oysters (exposed or control) were challenged with
291 *V. aestuarianus*.

292 The first mortalities were observed 96 hours post cohabitation with donor oysters (**Figure 3**).
293 Compared to control condition ($survival_{t=408h}$ = 0.14), a significant increase in survival was
294 observed at t = 408 hours post-cohabitation for oyster exposed to bacterial strains
295 *Pseudoalteromonas sp.* hCg-42 ($survival_{=408h}$ = 0.60 ; p-value < 0.001), *Yoonia sp.* THAU59
296 ($survival_{=408h}$ = 0.49 ; p-value < 0.001), *Bacillus sp.* ARG61 ($survival_{=408h}$ = 0.47 ; p-value =
297 0.004), and *Cytobacillus sp.* ARC29 ($survival_{=408h}$ = 0.40 ; p-value = 0.031). No difference in
298 survival was observed for oyster exposed to *Halomonas sp.* LTB66 ($survival_{=408h}$ = 0.19 ; p-
299 value = 1) or *Vibrio sp.* LTB1 ($survival_{=408h}$ = 0.07 ; p-value = 1) (**Figure 3**)(**Table 1**). A forest
300 plot analysis confirmed these results and indicates that a significant reduction of the mortality
301 risk of 70% (Log-Rank test: p-value < 0.001), 54% (Log-Rank test: p-value < 0.001), 46%
302 (Log-Rank test: p-value = 0.002) and 58% (Log-Rank test: p-value < 0.01) was observed for
303 the adult oysters exposed to the bacterial strains *Pseudoalteromoans sp.* hCg-42, *Bacillus sp.*
304 ARG61, *Cytobacillus sp.* ARC29 and *Yoonia sp.* THAU59 respectively
305 (**Supplementary_File_2 Figure S1**).

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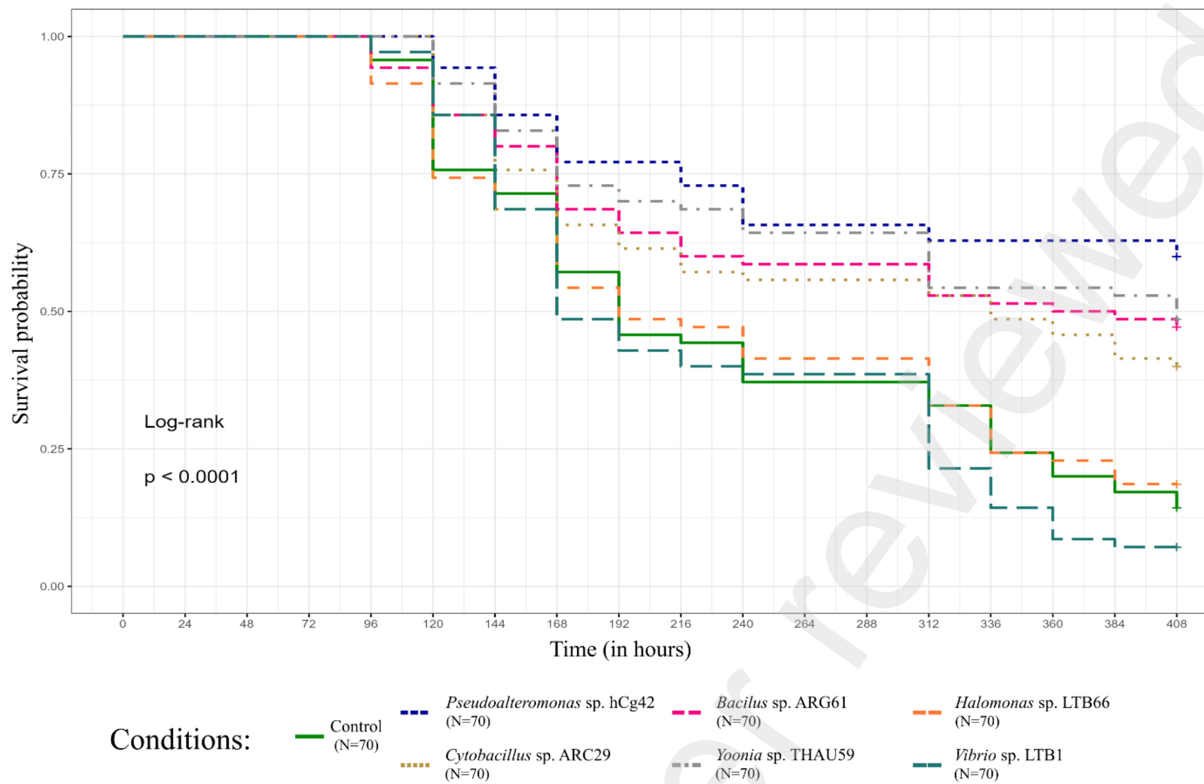

308

309 **Figure 3: Four bacterial strains have improved oyster survival against *V. aestuarianus***
310 **infection.**
311 Kaplan-Meier curve representing survival probability of oysters for the control (green solid
312 line) or exposed to candidate bacteria conditions during *Vibrio aestuarianus* infection.

313

314 **Table 1: Oyster survival data at t=408h post *V. aestuarianus* infection.**

Condition	survival (at t=408h)	std.err	p-value
Control	0.143	0.042	NA
Pseudoalteromonas sp. hCg-42	0.600	0.059	0.000001
Bacillus sp. ARG61	0.471	0.060	0.00398
Halomonas sp. LTB66	0.186	0.047	1.00000
Cytobacillus sp. ARC29	0.400	0.059	0.03107
Yoonia sp. THAU59	0.486	0.060	0.00032
Vibrio sp. LTB1	0.071	0.031	1.00000

315

316

317 **Addition of beneficial strains slightly modifies the bacterial alpha diversity of the oyster**
318 **microbiota**

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319 To test the immediate effect of the bacterial exposure on oyster microbiota, we analysed the
320 bacterial communities by 16S rRNA gene sequencing after seven days of bacterial exposure
321 with the last addition of bacteria performed 72 hours before sampling (**Figure 1**).

322 Sequencing of the V3-V4 hypervariable region of the 16S rRNA gene resulted in a total of
323 1,713,283 clusters for a total of 40 samples. After a quality control (deleting primers and low-
324 quality sequences, merging, and removing chimeras) and ASV clustering, 1,268,467 reads
325 (74%) with an average of 31,712 reads per sample were retained for downstream analyses.
326 Analysis of the alpha diversity metrics (richness, Shannon and Pielou) (**Figure 4**) indicates that
327 there are no significant differences for the species richness between the different conditions of
328 bacterial exposure (**Figure 4A**). However, we observed a greater range of diversity in the oyster
329 microbiota from the control (217 – 502), and those exposed to *Pseudoalteromonas sp.* hCg-42
330 (82 – 570) and to *Bacillus sp.* ARG61 (94 – 590) compared to *Halomonas sp.* LTB66 (289 –
331 429), *Cytobacillus sp.* ARC29 (262 – 595), *Yoonia sp.* THAU59 (267 – 535) or *Vibrio sp.* LTB1
332 (183 – 414) exposure. The alpha diversity with Shannon index (**Figure 4B**), which considers
333 taxon diversity and abundance, coupled with the Pielou index (**Figure 4C**) which considers the
334 distribution of individuals within species, show trends (NS Kruskal-Wallis test) where oysters
335 exposed to *Cytobacillus sp*. ARC29 (Shannon: 4.35 – 5.08; Pielou: 0.77 – 0.81) and *Yoonia sp.*
336 THAU59 (Shannon: 4.29 – 5.47; Pielou: 0.77 – 0.87) have more diverse bacterial communities
337 with a more equitable distribution of species compared to the control condition (Shannon: 3.65
338 – 4.88 ; Pielou: 0.68 – 0.80) or the condition exposed to *Pseudoalteromonas sp.* hCg-42
339 (Shannon: 2.86 – 5.22 ; Pielou: 0,64 – 0.82).

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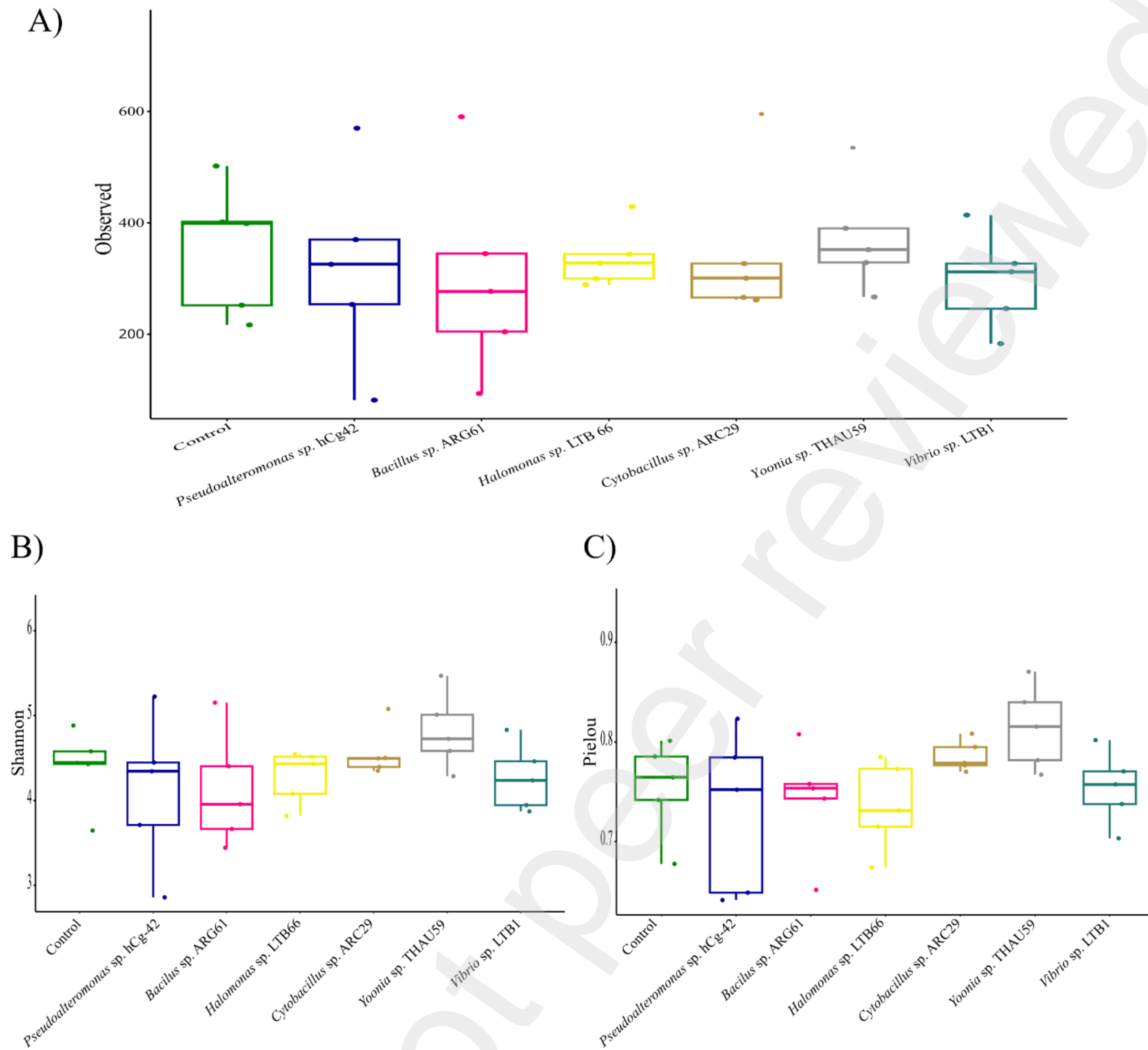

341

342 **Figure 3: Seven days of bacterial exposure did not induce significant changes in alpha**
343 **diversity.**
344 Boxplot representing the alpha diversity metrics (y axis) of oyster microbiota for control and
345 exposed to selected bacteria (n=5 oyster per conditions) with Observed (A), Shannon (B) and
346 Pielou (C) indices.

347

348 **Beneficial bacteria do not maintain in oyster tissues but significantly impact the beta**
349 **diversity of the bacterial microbiota**

350 After 7 days of exposure to the beneficial bacteria, we checked for their presence with the last
351 addition of bacteria performed 72 hours before sampling. Doing a blast search against all the
352 ASVs obtained using the 16S DNA sequences of the selected beneficial bacteria as a query, we
353 were not able to detect the administered bacteria in the microbiota of oysters
354 (**Supplementary_File_2 Figure S2A**) whereas the four bacteria were detected in the mock

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355 community although *Cytobacillus* and *Bacillus* were both affiliated to *Bacillus*
356 (**Supplementary_File_2_Figure_S2A**). The ASVs from the Mock were thus manually
357 reassigned to their bacterial strain in accordance with the BLAST results in particular for
358 *Bacillus sp.* ARG61 and *Cytobacillus sp.* ARC29 (**Supplementary_File_2_Figure_S2B**).

359

360 Dissimilarity analysis, based on the Bray-Curtis index, on adult oyster microbiota revealed
361 significant differences in microbiota composition between control oysters and oysters exposed
362 to *Pseudoalteromonas* sp. hCg-42, *Bacillus* sp. ARG61, *Halomonas* sp. LTB66, *Cytobacillus*
363 sp. ARC29, *Yoonia* sp. THAU59 and *Vibrio* sp. LTB1 (**Table 2**).

364

365 **Table 2: The beneficial bacterial exposure induces significant changes in the bacterial**
366 **composition of the exposed oysters.**
367 Results of permanova on the Bray-Curtiss dissimilarity matrix showing the effects of microbial
368 exposure on microbiota community composition compared to the control condition. Analyses
369 were carried out on five oysters per condition, excepted for oysters exposed to
370 Pseudoalteromonas sp. hCg-42, for which four oysters were used due to an abnormal sample
371 that was discarded from the analysis. The p-values were obtained using 100,000 permutations.

372

Conditions (Compared to control)	Dum Sq	R²	F	*p-value*
Pseudoalteromonas sp. hCg-42	0.32	0.23	2.09	0.008
Bacillus sp. ARG61	0.56	0.32	3.81	0.011
Halomonas sp. LTB66	0.56	0.35	4.23	0.013
Cytobacillus sp. ARC29	0.52	0.32	3.80	0.006
Yoonia sp. THAU59	0.35	0.20	2.04	0.008
Vibrio sp. LTB1	0.27	0.19	1.90	0.050

373

374

375 At the phylum level, control oysters were dominated by Proteobacteria (54.1%) and
376 Bacteroidota (23.2%) followed by Verrucomicrobiota (9.2%) and Firmicutes (3.6%) (**Figure**
377 **5A**). For oysters exposed to selected bacteria, Proteobacteria and Bacteroidota remained the
378 dominant phyla. In comparison with the control condition, oysters exposed to
379 *Pseudoalteromonas sp.* hCg-42 have a greater proportion of Firmicutes (13%) and
380 Fusobacteriota (12.9%). The other conditions displayed a relatively similar composition

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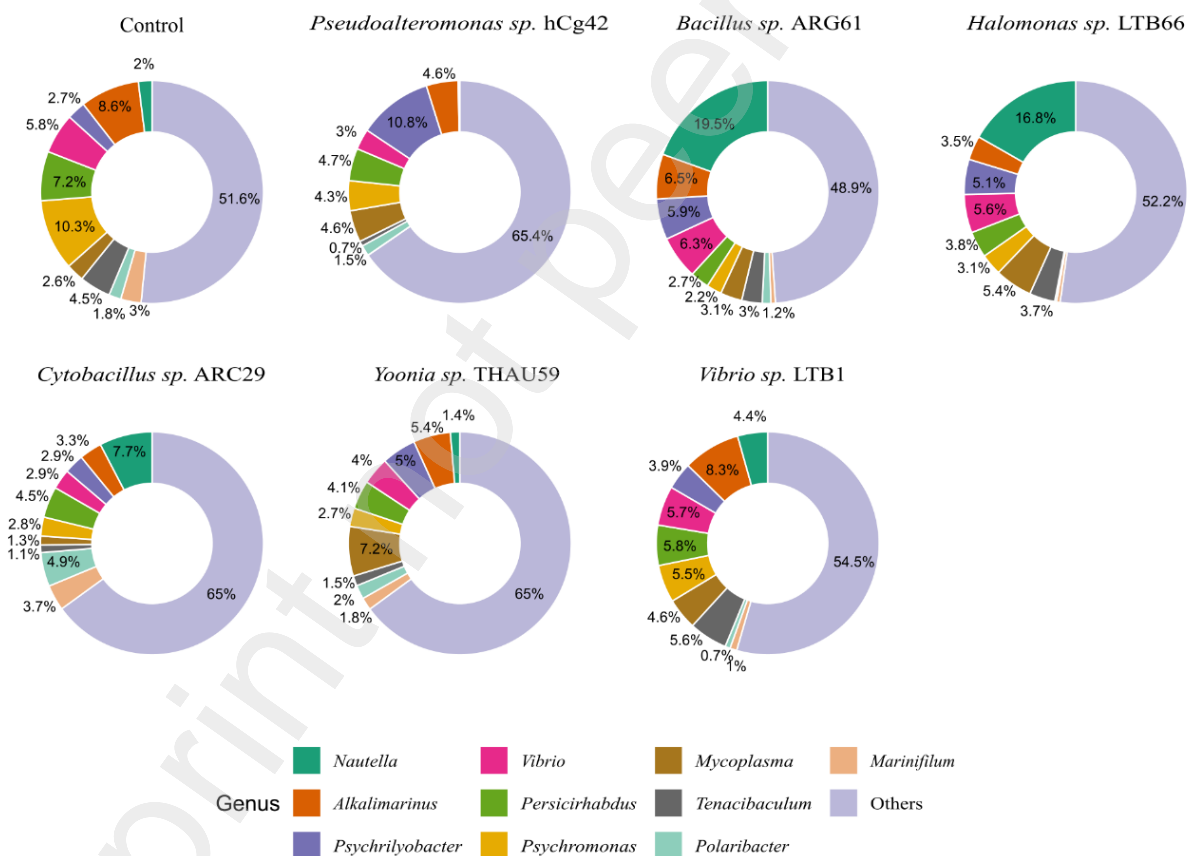
381 compared to control condition (**Supplementary_File_2 Figure S3**). At the Genus level, in the
382 control condition, the most abundant taxa were *Psychromonas* (10.3%), *Alkalimarinus* (8.6%),
383 *Persicirhabdus* (7.2%) and *Vibrio* (5.8%) (**Figure 5**). In comparison, for oysters exposed to
384 *Bacillus sp.* ARG61, *Halomonas sp.* LTB66 and *Cytobacillus sp.* ARC28 the most abundant
385 genera were *Nautella* (19.5, 16.8 and 7.7% respectively), *Alkalimarinus* (6.5, 3.5 and 3.3%),
386 *Psychrilyobacter* (5.9, 5.1 and 2.9%) and Vibrio (6.3, 5.6 and 2.9%). Oysters exposed to
387 *Pseudoalteromonas sp.* hCg-42 displayed a higher proportion of *Psychrilyobacter* (10.8%) and
388 *Mycoplasma* (4.6%). Oysters exposed to *Yoonia sp.* THAU59 displayed *Mycoplasma* (7.2%),
389 *Alkalimarinus* (5.4%) and *Psychrilyobacter* (5%) as most abundant genera. Last for oysters
390 exposed to *Vibrio sp.* LTB1 we identified *Alkalimarinus* (8.3%), *Persicirhabdus* (5.8%), *Vibrio*
391 (5.7%) *Tenacibaculum* (5.6%) and *Psychromonas* (5.5%) (**Figure 5**).

392



393

394 **Figure 4: Bacterial composition differs according to bacterial exposure.**
395 Donut plot representing the mean relative abundance of bacterial communities for the control
396 (n=5) or exposed to selected bacteria (n=5/condition) oyster samples at the genus level.

397

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398 **Discussion:**

399 Infectious diseases are a threat for oyster farming, and among them, the pathogenic bacterium
400 *V. aestuarianus* has been observed to spread across Europe (Mesnil et al. 2022). To ensure the
401 sustainability of oyster farming, it is crucial to develop effective, sustainable and socially
402 accepted strategies to fight vibriosis. In this study, we explored the antimicrobial potential of
403 bacteria isolated from oysters, with the aim of identifying natural antagonists that could mitigate
404 the harmful effects of *V. aestuarianus* infections in oyster farms.

405

406 **Oyster microbiota is a promising source of bacteria with antibacterial activity**

407 By screening a collection of 334 bacteria, we identified 76 bacteria (22.7% of the collection)
408 that displayed an antibacterial activity by co-culture, and eight of them (2.4% of the collection)
409 displaying a antibacterial activity in their supernatant against at least one of the target bacteria.
410 We acknowledge that we may have lost heat sensitive antimicrobial molecules since our
411 protocol to collect the supernatant include a heating step at 100°C. This could explain the small
412 proportion of effective molecules present in the supernatant fractions. Furthermore, the
413 spectrum of activity of the selected bacteria differed depending on if the antimicrobial tests
414 were performed using coculture assay or using the culture supernatant. This could be explained
415 by the release of an antibacterial compound during the centrifugation and/or the heating steps.
416 It also underlines that the methodology used for the assay is a key issue for antimicrobial
417 screening. Further tests using a different approach of supernatant extraction will therefore be
418 necessary to be as exhaustive as possible. Furthermore, it will be interesting to carry out
419 additional antibacterial activity tests using a broader range of pathogenic targets affecting
420 species of aquaculture interest. This will help to decipher if the observed antimicrobial activity
421 is restricted to oyster pathogens or if it can be expanded to applications to a broader range of
422 species of economic interest.

423

424 **Exposure to four bacterial strains (*Pseudoalteromonas sp.* hCg-42 ; *Bacillus sp.* ARG61 ;**
425 ***Cytobacillus sp.* ARC29 and *Yoonia sp.* THAU59) induced a significant reduction of the**
426 **mortality risk against *V. aestuarianus* infection.**

427 Four bacterial strains (*Pseudoalteromonas sp.* hCg-42 ; *Bacillus sp.* ARG61 ; *Cytobacillus sp.*
428 ARC29 and *Yoonia sp.* THAU59) among 6 tested induced a significant reduction, from 46% to

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429 70 %, of the mortality risk against *V. aestuarianus* infection. To our knowledge, this is the first
430 demonstration of a protective effect against *V. aestuarianus* by a potential antagonistic
431 bacterium. The Vibrio challenge was performed 72 hours after the last addition of the beneficial
432 bacteria, and it will be important to test longer term effect. Indeed, the dynamics of Vibrio
433 infection can be long and/or chronic-like (Travers et al. 2017). It is therefore possible that the
434 protection we observed only delayed the progression of the infection.

435 Further analyses are also required to determine the mechanisms underlying resistance to *V.*
436 *aestuarianus*. Here we did not detect the administered bacteria in the microbiota 72 hours after
437 it was added. The absence of detection suggests that the added bacteria are likely not present as
438 a major dominant strain in the microbiota. Our methodology was validated using a mock assay;
439 however, we cannot rule out the possibility that the added strain may be present as a minority
440 strain, which our method may have failed to detect due to a lack of sensitivity. Furthermore,
441 this absence of detection is not surprising since it was reported several times in the literature
442 that added probiotic strains do not maintain in their host microbiota. This can be observed in
443 the European abalone *Haliotis tuberculata* where exposure to the *Pseudoalteromonas* hCg-6
444 exogenous strain, result in only a temporary presence of the probiotic strain probiotic strain in
445 the haemolymph rather than the establishment of a long-term interaction (Offret et al. 2018).
446 Exposure of *M. gigas* larvae to bacteriocin-like inhibitory substance (BLIS)-producing
447 *Aeromonas*, showed that the probiotic strain concentration decreased right after it was added to
448 the oyster and was not detectable 72 h after its addition (Gibson et al. 1998). Furthermore,
449 among the four strains conferring a protective effect against *V. aestuarianus*, three bacteria
450 *(Bacillus sp.* ARG61 ; *Cytobacillus sp.* ARC29 and *Yoonia sp.* THAU59) had no antibacterial
451 activity against *V. aestuarianus in vitro*, making the explanation of resistance acquisition even
452 more complex. Taken together, these results suggest that a direct antagonistic effect of the
453 beneficial strains against *Vibrio* may likely not be at the origin of the resistance that we observe
454 in our experiments. The change in the microbiota composition could rather explain the observed
455 beneficial effect. Such an impact on microbiota composition after exposure to beneficial strains
456 has been reported in other species. This beneficial shift in microbiota composition following an
457 exposure to microorganisms has already been observed in Pacific oysters, where exposure to a
458 complete microbiota or mixes of cultivable bacteria led to changes in microbiota coupled with
459 improved survival against POMS infection (Fallet et al. 2022; Dantan et al. 2024b). This was
460 also observed for *Crassostrea virginica* oyster larvae, which, following an exposure to the
461 beneficial marine bacterium *Phaeobacter inhibens* S4, saw the composition of their microbiota

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462 modified, favouring in particular bacteria of the *Alteromonas* and *Pseudomonas* genus (Takyi
463 et al. 2024). Exposure of *C. sikamea* oysters to *Streptomyces* strains N7 and RL8 also induced
464 a shift in the diversity and composition of their microbiota, leading to a decrease in *Vibrio*
465 bacteria (García Bernal et al. 2017). An alternative still non-exclusive hypothesis is that the
466 exposure to these bacteria has induced immunostimulation effects as it has been previously
467 reported in oysters, abalone and scallops. Specifically, studies have demonstrated that an
468 exposure of *C. virginica* larvae to probiotic bacteria *Bacillus pumilus* RI06–95 and *Phaeobacter*
469 *inhibens* S4 led to improved survival in the face of *V. coralliilyticus* RE22 infection
470 simultaneously with an activation of immune signalling pathways (NF-kB and MAPK
471 pathways) and expression of immune effectors such as serine protease inhibitor (Cv-spi2),
472 mucins and antimicrobial histone H2B (Modak and Gomez-Chiarri 2020). Similarly, an
473 exposure of *M. gigas* larvae to a mix of probiotics bacteria, led to an increased expression of
474 immune signalling proteins (TOLL) and immune effectors such as interleukin IL-17 or MD88.
475 Furthermore, larvae exposed to this mix of probiotics showed an increased survival against *V.*
476 *coralliilyticus* infection (Hesser et al. 2024). Comparatively, *Argopecten purpuratus* scallop
477 larvae exposed to bacterial strains belonging to *Psychrobacter*, *Hydrogenophaga*, and
478 *Shewanella* genera displayed no mortality after 24h post infection to *V. bivalvicida* VPAP30.
479 This beneficial effect could be due to an immunomodulation of genes coding for opsonin,
480 superoxide dismutase (SOD), Toll-like receptor (TLR) or Lysozyme following the bacterial
481 exposure (Muñoz-Cerro et al. 2024). At last, New Zealand black-footed abalone *Haliotis iris*,
482 displayed a significantly increase in the number of total haemocytes count (HTC), non-
483 apoptotic cells, an higher percentage of ROS-positive cells and an higher viability following an
484 exposure to multi-strain probiotics (Grandiosa et al. 2018).

485

486 Conclusion:

487 In this study, we have shown that the administration of bacteria isolated from *M. gigas*
488 microbiota and displaying *in vitro* antibacterial activities against various pathogens or
489 opportunists, could increase the survival of oysters against *V. aestuarianus* infectious
490 challenges. Further studies will be needed to understand the immune molecular mechanisms
491 involved in the tolerance conferred by these bacteria. It would also be interesting to characterise
492 and purify molecules that are active against pathogens and opportunists. These molecules may
493 offer a promising way to mitigate the effects of these infectious diseases. A sustainable oyster
494 farming is a key to develop this growing industry in a context of global changes and emerging

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495 infectious diseases. The development of prophylactic methods such as the use of probiotics is
496 a necessity.

497

498 **Competing interests**

499 The authors declare that they have no competing interests.

500

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509

510 **Authors' contributions**

511 LDa, LDé, BM, BP, EM, GC and JVD contributed to oyster sampling. LDa, PC, YF, RL and
512 LI contributed to bacteria collection. LDa, and PC performed antibacterial activities tests. LDa,
513 LDé, BM, BP, and MM performed oyster experiments. LDa, JFA, CG, OR, JVD, CC and ET
514 prepared samples and performed DNA extraction from the oyster samples for analyses. LDa,
515 CC and ET performed microbiota analyses. LDa, LDé, BM, MAT, BP, MM, YF, YG, JVD,
516 CC and ET conceptualized and designed the experiments. LDa, CC and ET wrote the original
517 draft. LDa, YG, JVD, CC and ET were involved in funds acquisition. All authors have read and
518 approved the final manuscript.

519

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531

532 ## Availability of data and materials

533 Raw sequence data for 16S sequencing for metabarcoding analysis have been made available
534 through the SRA database (BioProject accession number PRJNA1183305, Link for reviewer:
535 https://dataview.ncbi.nlm.nih.gov/object/PRJNA1183305?reviewer=gjejj6ied1oultoo7v0usbu
536 hda)

537

538 ## Ethical approval

539 The animal (oyster *Magallana gigas*) testing followed all European regulations concerning
540 animal experimentation. The authors declare that the use of genetic resources fulfils the French
541 and EU regulations on the Nagoya Protocol on Access and Benefit-Sharing (French legislation
542 2019-486).

543

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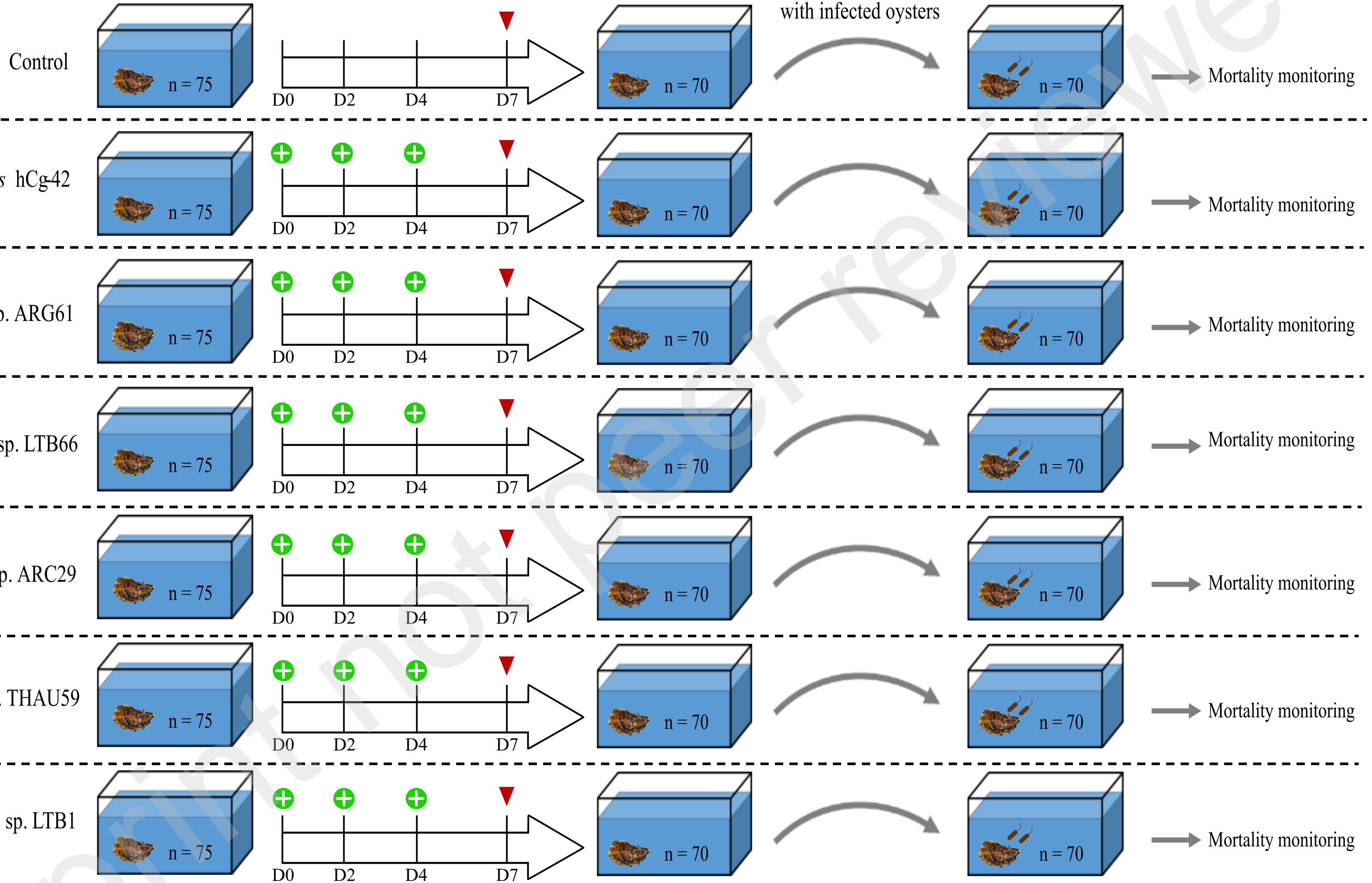

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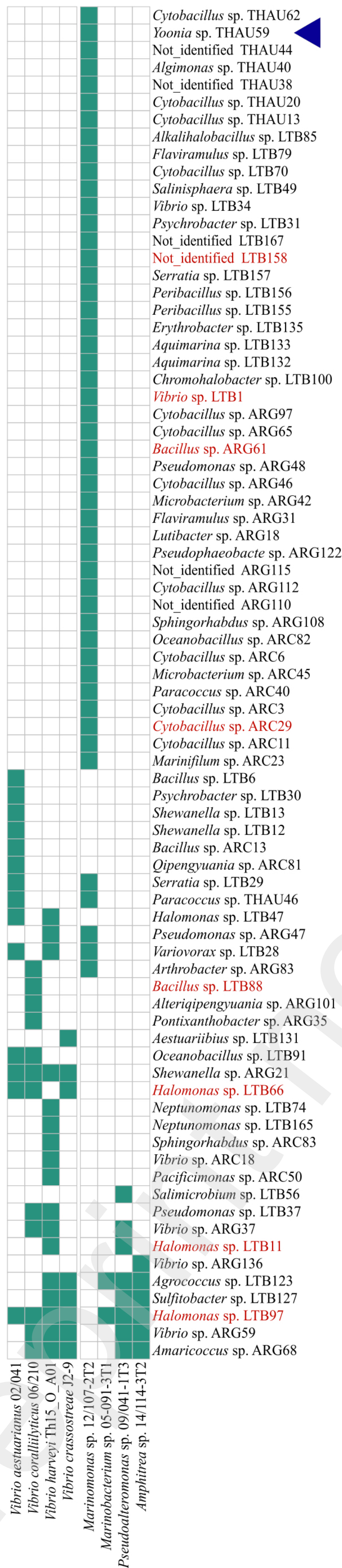

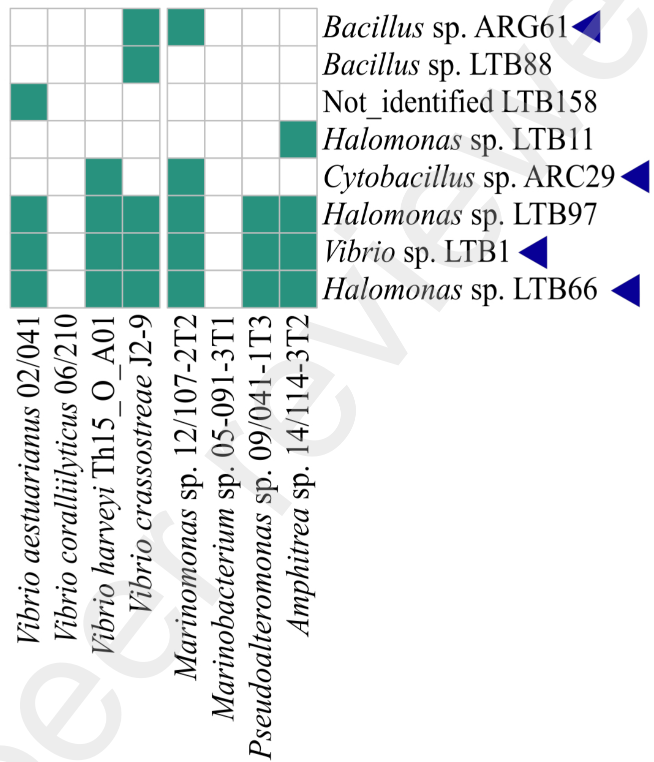

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1.00
0.75
0.50
0.25
0.00

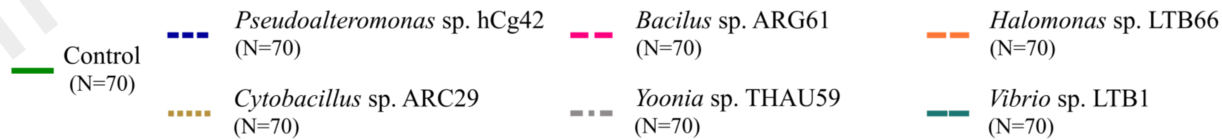
Survival probability

Log-rank

p < 0.0001

0 24 48 72 96 120 144 168 192 216 240 264 288 312 336 360 384 408

Time (in hours)



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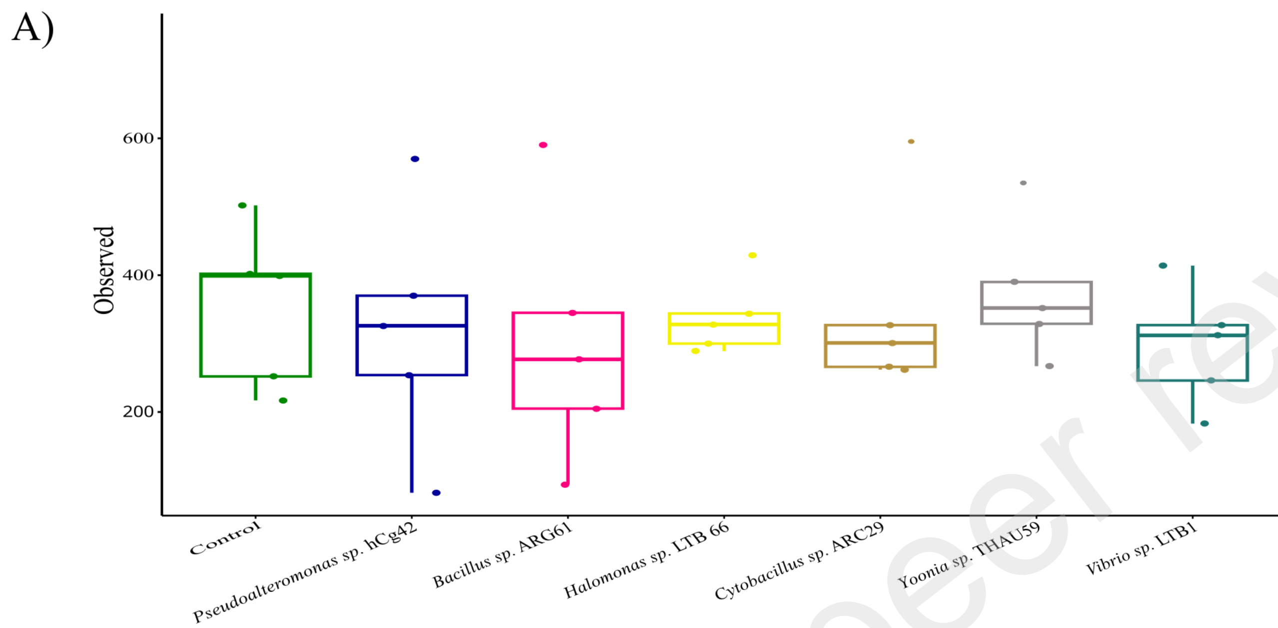

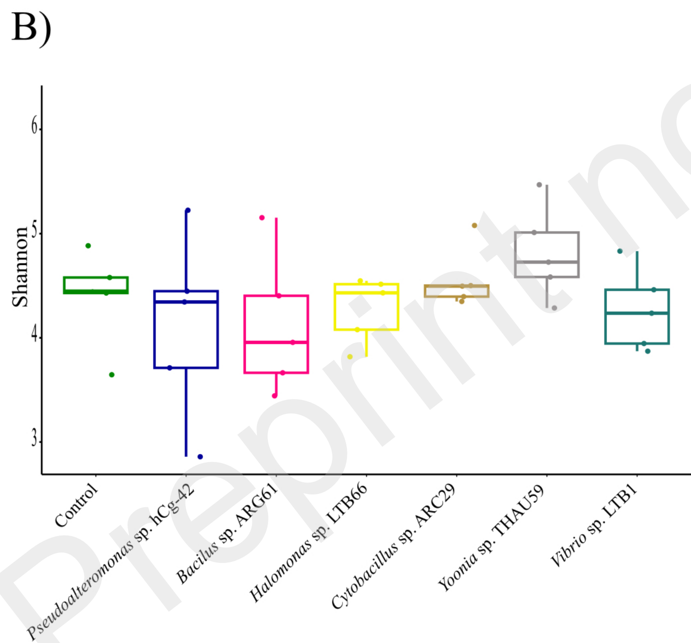

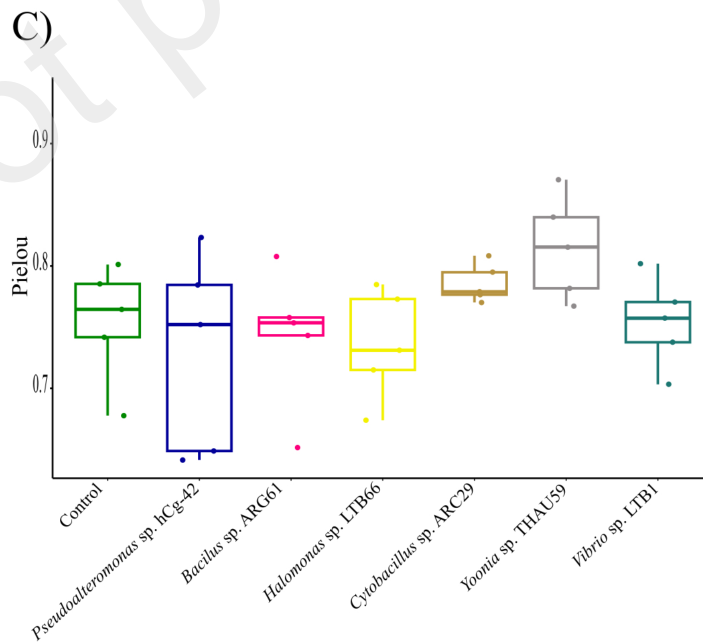

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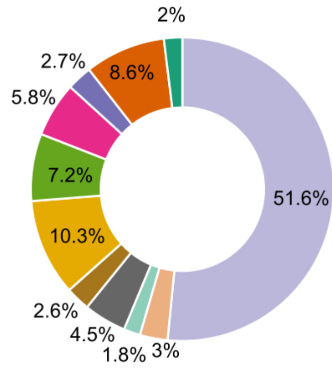
Control



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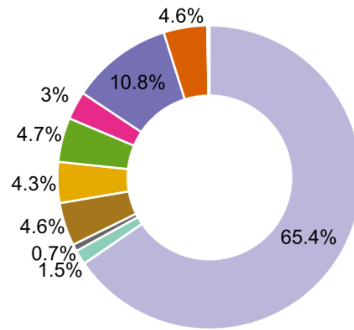
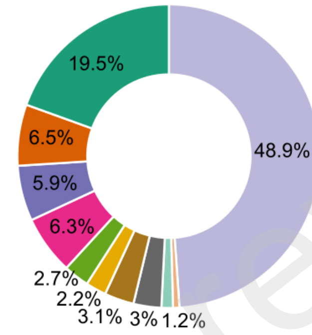
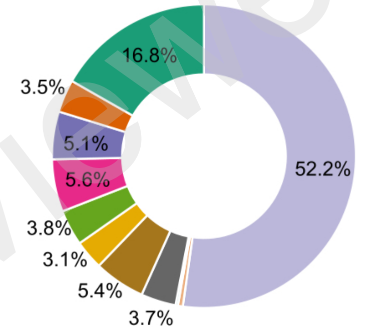


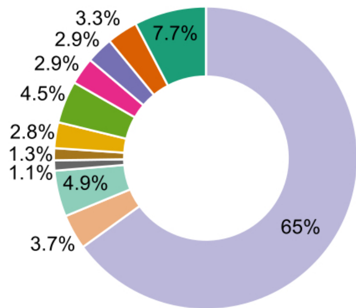
Bacillus sp. ARG61



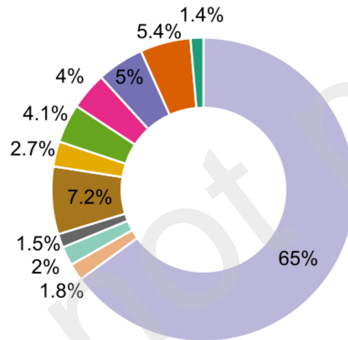
Halomonas sp. LTB66



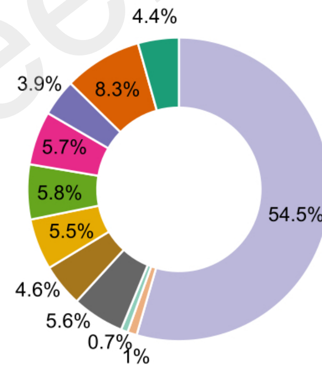
Cytobacillus sp. ARC29

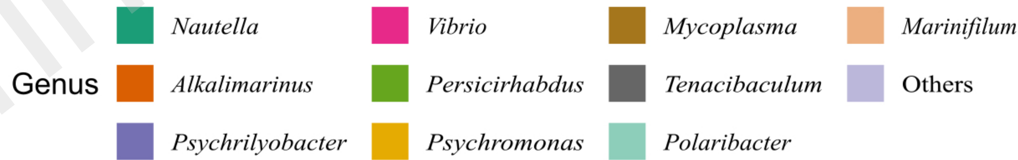


Yoonia sp. THAU59



Vibrio sp. LTB1





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