# **Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy)**

Vezzulli L[1,2]*, Stagnaro L[1,2], Grande C[1], Tassistro G[1], Canesi L[1], Pruzzo C[1]

[1] Department of Earth, Environmental and Life Sciences (DISTAV), University of Genoa, Genoa, Italy
[2]These authors contributed equally to this work

*corresponding author:
Luigi Vezzulli
Department of Earth, Environmental and Life Sciences (DISTAV), University of Genoa, Genoa, Italy
luigi.vezzulli@unige.it

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## Abstract

The pacific oyster *Crassostrea gigas* and the Mediterranean mussel *Mytilus galloprovincialis* are two widely farmed bivalve species which show contrasting behaviour in relation to microbial diseases, with *C. gigas* being more susceptible and *M. galloprovincialis* being generally resistant. In a recent study, we showed that different susceptibility to infection exhibited by these two bivalve species may depend on their different capability to kill invading pathogens (*e.g. Vibrio* spp.) through the action of haemolymph components. Specific microbial-host interactions may also impact bivalve microbiome structure and further influence susceptibility/resistance to microbial diseases. To further investigate this concept, a comparative study of haemolymph and digestive gland 16SrDNA gene-based bacterial microbiota profiles in *C. gigas* and *M. galloprovincialis* co-cultivated at the same aquaculture site was carried out using pyrosequencing. Bacterial communities associated with bivalve tissues (hemolymph and digestive gland) were significantly different from those of seawater, and were dominated by relatively few genera such as *Vibrio* and *Pseudoalteromonas*. In general, *Vibrio* accounted for a larger fraction of the microbiota in *C. gigas* (on average 1.7 fold in the haemolymph) compared to *M. galloprovincialis*, suggesting that *C. gigas* may provide better conditions for survival for these bacteria, including potential pathogenic species such as *V. aestuarianus*. *Vibrios* appeared to be important members of *C. gigas* and *M. galloprovincialis* microbiota and might play a contrasting role in health and disease of bivalve species. Accordingly, microbiome analyses performed on bivalve specimens subjected to commercial depuration highlighted the ineffectiveness of such practice in removing *Vibrio* species from bivalve tissues.

42 **Keywords:** *Mytilus galloprovincialis, Crassostrea gigas,* Next Generation Sequencing, 16SrDNA,
43 Microbiota
44

45 **Introduction**

46 Recent advance in DNA sequencing technology is enabling new insights into microbial community
47 diversity associated with human and animal tissues [1-3]. It is now recognized that host associated
48 microbial communities (õmicrobiotaö) are playing an important role in animal health by providing
49 multiple roles, ranging from nutrient processing to protection from diseases [4]. Marine
50 invertebrates host high microbial abundance and diversity [5], and alteration of the microbiota due
51 to stressful conditions and/or environmental changes was previously linked with a condition of a
52 compromised health status and susceptibility to diseases (*e.g.* through the rise of opportunistic
53 pathogens and/or colonization by non resident microbial species) [6,7].

54 Microbial communities associated with bivalve tissues were historically investigated by means of
55 culture-dependent methods [8,9], whilst only few studies have been conducted so far to estimate
56 bivalve microbiota using next generation sequencing (NGS) technology [7,10,11,12,13]. In Europe
57 mass mortality episodes of the pacific oyster (*Crassostrea gigas*) in farming areas were reported at
58 increasing frequency in recent years [14] and are attributed to complex interactions among oysters,
59 microbial pathogens and environmental variables [15]. For example, stressful environmental
60 conditions such as warm seawater temperatures were observed to favour shift of *C. gigas* bacterial
61 communities toward pathogen-dominated communities, also promoting colonization by secondary
62 opportunistic pathogens [7]. In contrast to *C. gigas*, other species of widely farmed bivalves such as
63 the Mediterranean mussel *Mytilus galloprovincialis* are generally not affected by mortality events
64 being less sensitive to changes in environmental conditions and microbial infections [16].
65 Unfortunately, molecular studies investigating *M. galloprovincialis* microbiota are lacking and, in
66 contrast to oysters, no information are currently available on the role that microbiome might play in
67 health and disease of farmed mussel [17].

68 We have recently shown that the different susceptibility to infection exhibited by *C. gigas* and *M.*
69 *galloprovincialis* may depend, at least in part, on their different capability to kill invading microbial
70 pathogens through the combined action of cellular (haemocyte) and soluble components of the
71 haemolymph [18]. In particular the extrapallial protein (EP) present in serum of *M.*
72 *galloprovincialis* (MgEP) but lacking in *C. gigas* has been recently shown to work as an opsonin
73 promoting D-mannose sensitive (MS) interactions of the bivalve pathogen *Vibrio aestuarianus*
74 01/032 strain and other bacteria (*e.g. Vibrio cholerae* ElTor N16961 and *Escherichia coli* MG1655)
75 carrying MS sensitive ligands with the hemocytes [19]. Presence/absence of this and/or similar

pathways driving microbial-host interactions in the haemolymph and other bivalve tissues may significantly impact microbiome structure and, in turn, influence susceptibility/resistance to microbial diseases in these animals.

Based on these previous observations, the aim of this study was to investigate whether differences exist in the overall microbiota structure of the two bivalve species that might partially be linked with their different susceptibility to microbial diseases. To this end, a comparative study of haemolymph and digestive gland bacterial microbiota profiles in *C. gigas* and *M. galloprovincialis* co-cultivated at the same aquaculture site was carried out using pyrosequencing techniques. The composition of bivalve microbial communities was also compared to that of ambient water where bivalve are grown and cultivated. As an additional objective of the study, microbiota analyses were performed in bivalve specimens that were subjected to commercial depuration, in order to investigate the impact of this practice on bivalve microbiota and its effectiveness in the removal of indigenous microbial pathogens.

## Material and methods

### Bivalve collection and preparation of haemolymph and digestive gland samples for molecular microbiological analysis

Adult mussels (*M. galloprovincialis* Lam) (n= 15, 5ó7 cm long) and oysters (*C. gigas*) (n= 15, 8ó10 cm long), were collected from the same batch in August 2015 from a shellfish farm located in the Gulf of La Spezia (44°04ø33øøN; 9°51ø20öE, Ligurian Sea, Italy). The site is the main shellfish production area in northwestern Italy. Mussels and oysters are farmed in co-cultivation by the suspended culture technique (rack culture method), with an estimated annual production of about 6.000 tons. Spat origin differs for the two bivalve species with *C. gigas* spat being mainly imported from Brittany (France) and *M. galloprovincialis* spat being naturally re-collected from the local cultivation area. Abnormal mortality of both species did not occur during the study period.

Additional mussels (n= 15, 5ó7 cm long) and oysters (n= 15, 8ó10 cm long) cultivated in the same area and period were obtained after depuration and treatment according to European and FAO regulations (bivalve are held in tanks of clean seawater for 24 hours; tanks seawater is treated through various steps to remove contaminants including sand filtration, UV treatment, ozonozation and biofiltration). 1 L of surface seawater (n=3) was also collected in the farming area by means of sterile plastic bottles. At the time of collection seawater temperature (26.7°C), salinity (39.8Ÿ ) and pH (8.2) were recorded.

In the laboratory all animals (depurated and non depurated) were cleaned of epibionts and gently washed with ASW to remove part of the non-resident microbiota. Haemolymph was extracted from

the posterior adductor muscle using a sterile 1 ml of syringe with an 18 G1/200 needle. With the needle removed, haemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 18°C. All individual animals were dissected under sterile conditions and digestive glands were removed, weighted and frozen for further microbiological analysis.

**Nucleic Acid Extraction**

Nucleic acids were extracted from pooled whole haemolymph (n=15) and digestive gland (n=15) samples with the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturerøs instructions. For water samples, 1 L of seawater was filtered on 0.2 Nucleopore filters (45 mm) and nucleic acids were extracted with the PowerWater DNA Isolation Kit (Mo Bio laboratories, inc), according to the manufacturerøs instructions. The amount of DNA extracted was determined fluorimetrically with QuantiFluorTM dsDNA System using a QuantiFluorTMfluorometer (Promega Italia srl, Milano, Italy).

**Real-Time PCR**

Real-time PCR for detection and enumeration of faecal indicators (*E. coli)*, bivalve (*V. aestuarianus*, *V. splendidus*-clade, *V. coralliilyticus*) and human (*V. cholerae, V. parahaemolyticus* and *V. vulnificus)* pathogenic bacteria in seawater and bivalve tissues were performed with the LightCyler (Roche Diagnostics, Mannheim, Germany) using primers and protocols reported in Table S1.

For *V. aestuarianus*, *V. splendidus*-clade, *V. cholerae, V. parahaemolyticus* and *V. vulnificus* a Taq-Man based PCR protocol was used (Table S1) [20-26]. Amplification reaction mixtures (20 L) contained the following: 1× TaqMan Master Mix (Roche Diagnostics), 200 nM primers, 25 nM probe, and DNA sample (0.262 ng/ L). Five microliters of DNA template was added to the reaction mixture. The PCR program used was as follows: initial denaturation at 95 °C for 10 min; subsequent 45 cycles of denaturation at 95 °C for 10 s; annealing at 60 °C [*V. aestuarianus and V.splendidus*-clade] for 15s, 59 °C [*V. cholerae*], 60 °C [*V. vulnificus*] or 59 °C [*V. parahaemolyticus*], for 20 s; and elongation at 72 °C for 1 s, followed by a cooling step at 40 °C for 30 s.

For *E. coli* and *V. coralliilyticus* a PCR protocol based on SYBR-Green I fluorescence was used (Table S1). Each reaction mixture contained 1×SYBR Green I Master Mix (Roche Diagnostics), 5.0 mmol of MgCl2 and 0.25 mmol of each primer in a final volume of 20 ul. The PCR programme was optimised as follows: initial denaturation at 95°C for 10 min, subsequent 40 cycles of denaturation at 95°C for 15 s [*E.coli* ] or for 5s [*V. coralliilyticus*], annealing at 60 °C for 30s [*E.coli.*] or 5 s

144 [*V.coralliilyticus*] and elongation at 72°C for 4 s**,** followed by a final elongation at 72°C for 10 min.

145 PCR runs were analyzed directly in the LightCycler using melting analysis and the software
146 provided with the instrument. For each single real-time PCR assay each DNA template was
147 analysed in triplicate (coefficient of variation 5%). The standards were prepared from pure nucleic
148 acid templates at known molar concentrations.

149

150 **16SrDNA Pyrosequencing**

151 A 16SrDNA PCR amplicon library was generated from genomic DNA extracted from bivalve
152 samples using the broad-range bacterial primers, 967f-5ø-CAACGCGAAGAACCTTACC-3ø and
153 1046r-5ø-CGACAGCCATGCANCACCT-3ø amplifying positions 965ó1063 (V6 hyper variable
154 region) of the *E. coli* numbering of the 16SrDNA [27]. Fusion primers were custom designed to
155 include the 16SrDNA complementary regions plus the Roche 454 A (5 -
156 GCCTCCCTCGCGCCATCAG-3 ) and B (5 -GCCTTGCCAGCCCGCTCAG-3 ) pyrosequencing
157 adapters and a specimen-specific barcode sequence. All primers were synthesized and HPSF or
158 HPLC purified by Tib Molbiol Srl (Genoa, Italy).

159 PCR products were cleaned using a Agencourt AMPure XP kit (Beckman Coulter s.r.l.) and
160 checked on an Agilent Bioanalyzer using a Agilent DNA 7500 kit (Agilent Technologies, Inc.).
161 Equal amounts ($1x10^9$ molecules/µl) of each product were then pooled together and finally adjusted
162 to $1x10^6$ molecules/µl. Amplicon libraries were bound to beads under conditions that favour one
163 fragment per bead and beads were emulsified in a PCR mixture in oil. After breaking the emulsion,
164 the DNA strands were denatured, and the beads carrying single-stranded DNA clones were
165 deposited into the wells on a PicoTiter- Plate (454 Life Sciences, Branford, CT, USA) for
166 pyrosequencing on a 454 GS Junior System (Roche, Basel,Switzerland). Sequence reads data were
167 archived at NCBI Sequence Read Archive (SRA) with accession number: SRP113304.

168

169 **Bioinformatics**

170 Bioinformatic analysis of NGS data was performed using the Microbial Genomics module (version
171 1.3) work-flow of the CLC Genomics workbench (version 9.5.1). After quality trimming based on
172 quality scores (quality nucleotide limit 0.05), trim of ambiguous nucleotides (n=2) and length
173 trimming, reads were clustered at 97% level of similarity into Operational Taxonomical Units
174 (OTUs). Chimera detection and removal was performed by the kmer searches pipeline of the
175 Microbial Genomics module (version 1.3). Ribosomal RNA gene reads were classified against the
176 non-redundant version of the SILVA SSU reference taxonomy (release 119; http://www.arb-
177 silva.de). Alpha diversity was calculated by rarefaction analyses of diversity measures (number of

178 total OTUs and bias-corrected form of Chao1 index) using the CLC software. Bray-Curtis
179 dissimilarity distance between each pair of samples were also calculated and Beta diversity was
180 estimated by applying Principal Coordinate Analysis (PCoA) on the resulting distance matrix.
181

## 182 Results

### 183 General sequencing results

184 A total of 408.783 amplicons were sequenced spanning the V6 hypervariable region of bacterial
185 rDNA gene from haemolpymph and digestive gland of *C. gigas* and *M. galloprovincialis*. To
186 minimise bias associated with random sequencing errors raw sequences were trimmed by
187 eliminating reads that contained one or more ambiguous bases, had errors in the barcode or primer
188 sequence, were atypically short (70 bp), and had an average quality score <30 [27]. This process
189 reduced the size of the whole dataset by ca 5%. To assess most abundant members of the
190 microbiota, phylogenetic identity of generated sequences from bivalve samples were analyzed using
191 the CLC Microbial Genomics Module (v.9.5.1). Trimmed reads were first clustered at 97%
192 similarity resulting in a total of 3.476 Operational Taxonomical Units (OTUs). Singletons (OTUs
193 with a single read in the data set) were excluded from the analysis. OTUs sequences were
194 BLASTed against SILVA reference database of nearly 227.000 high quality rDNA genes for the
195 prokaryotic kingdoms as described in the method session. The results of BLASTN were used to
196 estimate the taxonomic content of the data set, using SILVA taxonomy with the CLC software.
197 Only reads occurring at least 5 times in the trimmed data set were assigned to bacterial taxa and
198 included in the results. Following these steps classification against SILVA reference database
199 assigned 233.998 reads to the domain Bacteria while 65.982 remained unassigned.
200

### 201 Comparison of *C. gigas* and *M. galloprovincialis* microbiota

202 In general, the composition of the bacterial community in both haemolymph and digestive gland of
203 *C. gigas* and *M. galloprovincialis* was dominated by few OTUs accounting for the majority of reads
204 in the analyzed samples (*e.g.* on average, the three most abundant OTUs, excluding non classified
205 phylotypes, accounted for >40% of total bacterial diversity)
206 Alpha-diversity metrics (*e.g.* number of total OTUs and bias-corrected form of Chao1 index)
207 indicated that bacterial diversity and richness were higher in the haemolymph compared to the
208 digestive gland in both bivalve species (Figure 1). Although rarefaction curves computed for total
209 OTUs abundance failed to reach a plateau (indicating that more sequencing effort should be
210 required to detect additional phylotypes), rarefaction curves calculated for Chao1 index and other
211 comparable biodiversity indices (*e.g.* Simpson's index and Shannon entropy, data not shown)

reached a stable value suggesting that sequencing depth was good enough to measure and compare alpha-diversity metrics in all samples (Figure 1).

In the haemolymph, bacterial diversity was higher in *C. gigas* than in *M. galloprovincialis* and was dominated by the genera *Pseudoalteromonas* ( 24% and 25% relative abundance in *C. gigas* and *M. galloprovincialis,* respectively*)* and *Vibrio* ( 24% and 14% relative abundance in *C. gigas* and *M. galloprovincialis,* respectively) (Figure 2). The *Vibrio* genus also dominated the composition of the bacterial community in the digestive gland, with higher relative abundance in *C. gigas* ( 36%) than *M. galloprovincialis* ( 28%) (Figure 2). In contrast, bacteria belonging to the genus *Pseudoalteromonas* accounted for a smaller fraction of the bacterial community in the digestive gland in both bivalve species (on average less than 8% of total bacterial abundance). Interestingly, the bacterial community associated with the digestive gland of *M. galloprovincialis* was dominated by the genus *Desulfovibrio* (a bacterial genus not detected in *C. gigas* samples), that showed a relative abundance greater than 40% in these samples (Figure 2). An additional large fraction of bacterial communities was represented by unclassified OTU sequences representing on average 20.1% ± 6.5% of the total bacteria diversity (Figure 2).

Bacterial community composition of ambient seawater differed substantially from bivalve bacterial communities. In the water compartment, unclassified phylotypes made up a significant fraction of the microbial community composition (38%), followed by the phylum *Cyanobacteria* (28%) and bacteria belonging to the *Rhodospirillaceae* (14%) family (Figure 2). In contrast, dominant phylotypes observed in bivalve tissues represent only a minor fraction of the water microbial community (*e.g.* 2.5% and 0.3% relative abundance for *Vibrio* and *Pseudoalteromonas,* in seawater, respectively) (Figure 2).

Distinct bacterial communities in seawater and bivalve tissues was also evident from the analysis of beta-diversity (Figure 3). Beta-diversity analysis also showed that the composition of the bacterial community was very similar in the haemolymph of *C. gigas* and *M. galloprovincialis*, whereas differences were observed in the bacterial community composition of digestive gland in the two bivalve species (Figure 3).

**Effect of depuration on *C. gigas* and *M. galloprovincialis* microbiota**

The composition and diversity of the bacterial community were also evaluated in haemolymph and digestive gland of specimens of *C. gigas* and *M. galloprovincialis* collected after commercial depuration. In general, depurated bivalve samples showed lower diversity and richness of associated bacterial populations when compared with non-depurated samples, as clearly showed by alpha-diversity metrics (Figure 1). In addition, both haemolymph and digestive gland samples from

246 depurated bivalves clustered together in PCoA analysis, indicating a high level of similarity of
247 bacterial diversity in these samples that differed significantly from beta-diversity metrics observed
248 for non-depurated samples (Figure 3).
249 A major shift in bivalve associated bacterial community structure following depuration practices
250 regarded *Vibrio* populations that were found to significantly increase both in *C. gigas* and *M.*
251 *galloprovincialis* tissues, with the only exception of *C. gigas* digestive gland (Figure 2). In the
252 haemolymph, the relative abundance of these bacteria increased from 24% to 42% in *C. gigas* and
253 from 14% to 56% in *M. galloprovincialis*. A significant increase in the relative contribution of
254 *Vibrio* to the whole bivalve bacterial community was also observed in the digestive gland of *M.*
255 *galloprovincialis* , where this bacteria increased from 29% to 54% following depuration.
256 *Pseudoalteromonas*, the dominant bacterial genus in the haemolymph of the two bivalve species
257 was found to significantly decrease in *M. galloprovincialis* following depuration (relative
258 abundance in the haemolymph from 25% to 9% before and after depuration, respectively) whilst it
259 remained fairly constant in *C. gigas* samples (relative abundance in the haemolymph from 24% to
260 29% before and after depuration, respectively) (Figure 2). Other major effects of depuration
261 observed on bivalve microbiota were linked to a significant increase in the relative abundance of the
262 genus *Stenotrophomonas* in the digestive gland of both *C. gigas* (relative abundance from <1% to
263 23% before and after depuration, respectively) and *M. galloprovincialis* (relative abundance from
264 <1% to 26% before and after depuration, respectively) and to the disappearance of *Desulfovibrio*
265 (relative abundance from 43% to 0% before and after depuration, respectively) in *M.*
266 *galloprovincialis* digestive gland (Figure 2).
267
268 **Indigenous potential pathogenic *Vibrio* species in *C. gigas* and *M. galloprovincialis* tissues**
269 Considering that the *Vibrio* genus represents a dominant group in bivalve tissues and play a role in
270 bivalve disease, the presence of potential pathogenic *Vibrio* species, was investigated by Real-Time
271 PCR in all samples (Table 1). The presence of *Vibrio* species pathogenic for humans and the fecal
272 indicator *E. coli* were also assessed (Table 1).
273 The bivalve pathogen *V. aestuarianus* was found in oyster haemolymph, but not in oyster digestive
274 gland and in *M. galloprovincialis* samples. *V. coralliilyticus*, another potential bivalve pathogen,
275 albeit found in seawater, was not detected in bivalve tissues. In contrast, bacteria belonging to the
276 *Vibrio splendidus* clade, which includes a number of potential pathogenic species for bivalves (*e.g.*
277 *Vibrio splendidus* and *Vibrio crassostrea*), were consistently found in *C. gigas* and *M.*
278 *galloprovincialis* tissues at concentration ranging from 9.2 $x10^3$ to 6.4 $x10^4$ genomic unit (GU)/g

279 (Table 1). Human pathogenic vibrio species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*
280 were never detected in bivalve samples.
281 Depuration practices did not reduce the concentration of indigenous pathogenic vibrios (Table 1). In
282 contrast, *V. aestuarianus* concentration increased from $2.4x10^2$ cell/ml to $6.6x10^2$ GU/g in the
283 haemolymph of *C. gigas* following depuration. Likewise, concentration of bacteria belonging to the
284 *V. splendidus* clade increased up to 3-fold in depurated samples as compared with non-depurated
285 ones. The indicator bacteria *E. coli* used as control of the efficiency of depuration was only found in
286 non depurated bivalves (6.3 $x10^2$ GU/g and 8.7 $x10^2$ GU/g in digestive gland of *C. gigas* and *M.*
287 *galloprovincialis*, respectively).
288
289 **Discussion**
290 **Comparison of *C. gigas* and *M. galloprovincialis* microbiota**
291 In this work, for the first time to our knowledge, the microbiota structure in haemolymph and
292 digestive gland of two contrasting bivalve species (*C. gigas* and *M. galloprovincialis*) that show
293 different susceptibility to microbial diseases was evaluated by 16SrDNA gene amplification and
294 pyrosequencing. Such comparison was made possible by taking advantage of co-cultivation of
295 oysters and mussels in a Mediterranean fish farm where both species are grown under the same
296 environmental conditions. The study was carried out during the warmest month of the year (August,
297 SST 26.7°C at the time of sampling), when susceptibility to diseases by bivalve species is generally
298 highest.
299 Microbial communities associated with bivalves were significantly different from those of seawater,
300 indicating the existence of a host-specific microbiota in bivalve tissues. Surprisingly, the
301 microbiota associated to the haemolymph of the two bivalve species showed a high degree of
302 similarity and it was dominated by the genera *Vibrio* and *Pseudoalteromonas*, both accounting on
303 average for more than a third of the total bacterial community. These genera were reported in
304 previous studies to represent dominant members of oyster microbiota [8, 28-30]. This not only
305 indicates that these genera are able to persist in the haemolymph environment, and therefore to
306 survive the antibacterial activity of haemolymph components, but also suggests that they might also
307 contribute to bivalve antimicrobial defence for example through the production of antimicrobial
308 compounds. Accordingly, *Pseudoalteromonas* species are generally found in association with
309 marine eukaryotes and display anti-bacterial, bacteriolytic, agarolytic and algicidal activities [31].
310 Vibrios are also commonly found in association with aquatic plants and animals, to which they
311 may provide a chemical defense for the host [32]. However, the *Vibrio* genus also include
312 bivalve pathogenic species, some of which are known to be associated with abnormal mortality

313 outbreaks in farmed animals (*e.g. V. aestuarianus* and *V. splendidus* clade in farmed oysters).
314 Interestingly, vibrios accounted for a larger fraction of the haemolymph microbiota in *C. gigas*
315 compared to *M. galloprovincialis* (on average 1.7 fold), and the pathogen *V. aestuarianus* was
316 detected by PCR in the haemolymph of *C. gigas*, but not in that of *M. galloprovincialsis* (Table 1).
317 This is in line with the lower susceptibility of mussels, with respect to oysters, to pathogenic *Vibrio*
318 species also in relation to the presence in mussels of specific hemolymph soluble components
319 (MgEP) that mediate adhesion, internalization and killing of *V. aestuarianus* by the hemocytes [18-
320 19, 33]. Overall, these results indicate that *Vibrio* might play a contrasting role in health and disease
321 of different bivalve species.
322 In contrast to haemolymph, microbial communities in digestive gland displayed a more diversified
323 composition in the two bivalves (Figure 2). *Vibrio* still represented a significant fraction of the
324 microbiota, whilst bacteria belonging to the *Pseudoalteromonas* genus were far less abundant in this
325 tissue. As found in the haemolymph, *Vibrio* contribution to the overall microbial community
326 structure was greater in *C. gigas* than in *M. galloprovincialis,* further supporting the hypothesis that
327 the oyster environment may provide better conditions for survival of these bacteria. From a
328 functional perspective, the bivalve digestive gland plays a key role in the intracellular and
329 extracellular digestion and storage of nutrients [34], and bacteria associated with this tissue may
330 also contribute to this role. In this light, *Desulfovibrio* found in association with the mussel
331 digestive gland is of particular interest, as bacteria belonging to this genus are capable to conduct
332 sulphate respiration under strict anaerobic conditions [35]. Sulphate-reducing bacteria (SRB) are
333 widespread in anaerobic environments, including the gastrointestinal tract of humans and other
334 animals [35] (anaerobic conditions may establish in bivalve grow-out bags especially when bivalve
335 are cultivated at high density as in the case of mussels). A potential role of these bacteria in
336 anaerobic energy metabolism of *M. galloprovincialis* might thus be envisaged. In particular, they
337 may contribute to the well-known capability of mussels to tolerate extended periods of hypoxia and
338 anoxia, which may be induced by shell valve closure during emersion, depletion of oxygen in the
339 surrounding water and other stressful conditions [36]. However, this remain a speculation and
340 studies are in progress to confirm such observations and evaluate the role of SRB in mussel
341 metabolism. It is finally worth noting that an additional large fraction of the haemolymph and
342 digestive gland microbiota in the two bivalves is represented by unidentified microorganisms and
343 rare taxa the role of which is yet to be assessed.
344
345 **Effect of depuration on *C. gigas* and *M. galloprovincialis* microbiota**

346 Depuration is a process by which bivalves are held in tanks of clean seawater under conditions
347 which maximize the natural filtering activity enabling the removal of microbial and other
348 contaminants before commercialization [37]. This process is used after collection and prior to
349 commercialization of farmed bivalves resulting in a major decrease of coliforms and other transient
350 bacteria in bivalve tissues. Albeit it is generally accepted that depuration practices are effective in
351 removing allochthonous bacteria (*e.g.* enteric microorganisms) they are generally considered only
352 partly effective, or ineffective, in removing other microbial contaminants such as naturally
353 occurring marine vibrios [37].
354 Accordingly, the results of Real time-PCR indicate that *E. coli*, generally utilized as a marker of
355 fecal contamination [38-39], was completely removed from mussel and oyster tissues following
356 depuration (Table 1). In contrast, indigenous *Vibrio* species (*e.g. Vibrio aestuarianus* and *V.*
357 *splendidus* clade) were unaffected by the depuration treatment (Table 1). Although the existence of
358 a host-specific microbiota in bivalves is still questioned, *Vibrio* species were found to represent a
359 dominant fraction of both *C. gigas* and *M. galloprovincialis* microbiota (see previous paragraph).
360 This observation suggests that these bacteria probably have a long co-evolutionary history with
361 their invertebrate hosts, probably representing permanent residents of the microbiota, which in turn
362 may lead to an increased resistance (persistence) to depuration. Interestingly, bacteria belonging to
363 the *Vibrio* genus not only were unaffected by depuration practice but also tend to increase in
364 abundance in depurated oysters and mussels when compared to non depurated ones. Such findings
365 might be better interpreted if we consider the more general influence that depuration had on the
366 bivalve microbiota structure. In fact, the most prominent effect of depuration was a significant loss
367 of microbial diversity in all samples from both bivalve species (Figure 1). Phylotype loss was
368 particularly evident for rare microbial taxa and, in turn, lead to a re-organization of dominant
369 bacterial genera within the bivalve microbiota (Figure 2). The increased dominance of bacteria
370 belonging to the *Vibrio* genus within the overall bacterial community associated to both
371 haemolymph and digestive gland of oysters and mussels may be probably related to new
372 environmental niches that are made available through microbiota re-organization following
373 depuration. Such an event is in line with the opportunistic nature of these bacteria, as also observed
374 in other marine invertebrates for *Vibrio* pathogenic species during infection. For example, alteration
375 of the coral microbiome under environmental stressful conditions, leading to decrease in dominant
376 holobiont members such as the genus *Endozoicomonas*, has been linked to coral susceptibility to
377 disease and infection by the coral pathogen *Vibrio coralliilyticus* [40-41]. Similarly to vibrios, other
378 bacterial groups such as the genus *Stenotrophomonas,* previously observed in association with
379 oysters [42], significantly increased in dominance in the digestive gland of *C. gigas* and *M.*

*galloprovincialis* following depuration. In contrast, bacterial group such as *Desulfovibrio* completely disappeared in *M. galloprovincialis* digestive gland probably in relation with the changing oxygen environment in depuration tanks.

Overall, these results show that depuration significantly affects bivalve microbiota. However, since this process may also favour opportunistic members of the bacterial community such as vibrios, this practice results ineffective in the removal of these indigenous pathogens from bivalve tissues. Alternative or additional protocols of depuration might thus be developed to challenge the threat posed by these pathogens in edible bivalves. For instance, settings of environmental conditions in depuration plants favoring hemolymph-mediated bacterial killing (e.g. increasing the production of serum opsonins) [19] and phage therapy [43] have been proposed. However, none of these practices has yet been tested and proved to be successful in real aquaculture depuration facilities.

## Conclusions

The result obtained in this work provide some points of novelty. First, they represent to our knowledge the first description of 16SrDNA gene-based microbiota profiles of the bivalve *M. galloprovincialis.* They also provide the first data on the comparison of microbiota profiles in two aquacultured bivalves (*C. gigas* and *M. galloprovincialis*) showing different susceptibility to microbial diseases. They finally represent the first NGS data on the effect of depuration on the bivalve microbiota.

The results underline both similarities and differences in the composition of the microbiota in different tissues (hemolymph and digestive gland) of oyster and mussels. In general bivalve bacterial community appeared to be host-specific and may have contrasting role (*e.g. Vibrio* spp.) in health and disease of bivalve species. Depuration was found to significantly affect bivalve microbiota; however, it must be taken into account that this process may also favour opportunistic members of the bacterial community such as vibrios. Overall, understanding microbiota-host interactions in farmed bivalves remains an open challenge with the potential to bring new essential knowledge in the field of biology and control of bivalve infectious diseases.


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607 **Figure 1**
608 Rarefaction curves for alpha-diversity metrics (number of total OTUs (a) and bias-corrected form of
609 Chao1 index (b)) calculated from 16rDNA gene-based profiling analysis of the bacterial community
610 in seawater and bivalve tissues (SW=seawater, HM=*M. galloprovincialis* haemolymph, HO=*C.*
611 *gigas* haemolymph, GM=*M. galloprovincialis* digestive gland, GO= *C. gigas* digestive gland,
612 DEP=depurated samples).
613
614 **Figure 2**
615 Relative abundances of bacterial genera found in seawater and bivalve tissues by 16rDNA gene-
616 based profiling analysis (SW=seawater, HM=*M. galloprovincialis* haemolymph, HO=*C. gigas*
617 haemolymph, GM=*M. galloprovincialis* digestive gland, GO= *C. gigas* digestive gland,
618 DEP=depurated samples).
619
620 **Figure 3**
621 3D PCoA plots of beta diversity for seawater and bivalve samples calculated using Principal
622 Coordinate Analysis (PCoA) applied on Bray-Curtis distance matrix (SW=seawater, HM=*M.*
623 *galloprovincialis* haemolymph, HO=*C. gigas* haemolymph, GM=*M. galloprovincialis* digestive
624 gland, GO=*C. gigas* digestive gland, DEP=depurated samples).
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