

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)

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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.

Keywords *Mytilus galloprovincialis* · *Crassostrea gigas* · Next generation sequencing · 16SrDNA · Microbiota

Introduction

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

Microbial communities associated with bivalve tissues were historically investigated by means of culture-dependent methods [8, 9], while only few studies have been conducted so far to estimate bivalve microbiota using

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next generation sequencing (NGS) technology [7, 10–13]. In Europe mass mortality episodes of the pacific oyster (*Crassostrea gigas*) in farming areas were reported at increasing frequency in recent years [14] and are attributed to complex interactions among oysters, microbial pathogens and environmental variables [15]. For example, stressful environmental conditions such as warm seawater temperatures were observed to favour shift of *C. gigas* bacterial communities toward pathogen-dominated communities, also promoting colonization by secondary opportunistic pathogens [7]. In contrast to *C. gigas*, other species of widely farmed bivalves such as the Mediterranean mussel *Mytilus galloprovincialis* are generally not affected by mortality events being less sensitive to changes in environmental conditions and microbial infections [16]. Unfortunately, molecular studies investigating *M. galloprovincialis* microbiota are lacking and, in contrast to oysters, no information are currently available on the role that microbiome might play in health and disease of farmed mussel [17].

We have recently shown that the different susceptibility to infection exhibited by *C. gigas* and *M. galloprovincialis* may depend, at least in part, on their different capability to kill invading microbial pathogens through the combined action of cellular (hemocyte) and soluble components of the haemolymph [18]. In particular the extrapallial protein (EP) present in serum of *M. galloprovincialis* (MgEP) but lacking in *C. gigas* has been recently shown to work as an opsonin promoting D-mannose sensitive (MS) interactions of the bivalve pathogen *Vibrio aestuarianus* 01/032 strain and other bacteria (e.g., *Vibrio cholerae* EITor N16961 and *Escherichia coli* MG1655) 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) ($n = 15$, 5–7 cm long) and oysters (*C. gigas*) ($n = 15$, 8–10 cm long), were collected from the same batch in August 2015 from a shellfish farm located in the Gulf of La Spezia (44° 04' 33" N; 9° 51' 20" E, 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 ($n = 15$, 5–7 cm long) and oysters ($n = 15$, 8–10 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 h; tanks seawater is treated through various steps to remove contaminants including sand filtration, UV treatment, ozonation, 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 (39.8‰) 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, hemolymph 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 manufacturer's 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 manufacturer's instructions. The amount of DNA extracted was determined fluorimetrically with QuantiFluor™ dsDNA

System using a QuantiFluor™ fluorometer (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 LightCycler (Roche Diagnostics, Mannheim, Germany) using primers and protocols reported in ESM 1: Table S1.

For *V. aestuarianus*, *V. splendidus*-clade, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* a Taq-Man-based PCR protocol was used (ESM S1: Table S1) [20–26]. Amplification reaction mixtures (20 µL) contained the following: 1 × TaqMan Master Mix (Roche Diagnostics), 200 nM primers, 25 nM probe, and DNA sample (0.2–2 ng/µL). 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 15 s, 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 (ESM S1: 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 µL. The PCR programme was optimized 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 5 s [*V. coralliilyticus*], annealing at 60 °C for 30 s [*E. coli*] or 5 s [*V. coralliilyticus*] and elongation at 72 °C for 4 s, followed by a final elongation at 72 °C for 10 min. PCR runs were analysed directly in the LightCycler using melting analysis and the software provided with the instrument. For each single real-time PCR assay each DNA template was analysed in triplicate (coefficient of variation 5%). The standards were prepared from pure nucleic acid templates at known molar concentrations.

16SrDNA Pyrosequencing

A 16SrDNA PCR amplicon library was generated from genomic DNA extracted from bivalve samples using the broad-range bacterial primers, 967f-5'-CAACGCGAAGAACC TTACC-3' and 1046r-5'-CGACAGCCATGCANACCT-3' amplifying positions 965–1063 (V6 hyper variable region) of the *E. coli* numbering of the 16SrDNA [27]. Fusion primers were custom designed to include the 16SrDNA complementary regions plus the Roche 454 A (5'-GCCTCCCTCGGCC

ATCAG-3') and B (5'-GCCTTGCCAGCCCCGCTCAG-3') pyrosequencing adapters and a specimen-specific barcode sequence. All primers were synthesized and HPSF or HPLC purified by Tib Molbiol Srl (Genoa, Italy).

PCR products were cleaned using a Agencourt AMPure XP kit (Beckman Coulter s.r.l.) and checked on an Agilent Bioanalyzer using a Agilent DNA 7500 kit (Agilent Technologies, Inc.). Equal amounts (1 × 10⁹ molecules/µL) of each product were then pooled together and finally adjusted to 1 × 10⁶ molecules/µL. Amplicon libraries were bound to beads under conditions that favour one fragment per bead and beads were emulsified in a PCR mixture in oil. After breaking the emulsion, the DNA strands were denatured, and the beads carrying single-stranded DNA clones were deposited into the wells on a PicoTiter- Plate (454 Life Sciences, Branford, CT, USA) for pyrosequencing on a 454 GS Junior System (Roche, Basel, Switzerland). Sequence reads data were archived at NCBI Sequence Read Archive (SRA) with accession number: SRP113304.

Bioinformatics

Bioinformatic analysis of NGS data was performed using the Microbial Genomics module (version 1.3) work-flow of the CLC Genomics workbench (version 9.5.1). After quality trimming based on quality scores (quality nucleotide limit 0.05), trim of ambiguous nucleotides ($n = 2$) and length trimming, reads were clustered at 97% level of similarity into Operational Taxonomical Units (OTUs). Chimera detection and removal was performed by the kmer searches pipeline of the Microbial Genomics module (version 1.3). Ribosomal RNA gene reads were classified against the non-redundant version of the SILVA SSU reference taxonomy (release 119; <http://www.arb-silva.de>). Alpha diversity was calculated by rarefaction analyses of diversity measures (number of total OTUs and bias-corrected form of Chao1 index) using the CLC software. Bray-Curtis dissimilarity distance between each pair of samples were also calculated and Beta diversity was estimated by applying principal coordinate analysis (PCoA) on the resulting distance matrix.

Results

General Sequencing Results

A total of 408,783 amplicons were sequenced spanning the V6 hypervariable region of bacterial rDNA gene from haemolymph and digestive gland of *C. gigas* and *M. galloprovincialis*. To minimize bias associated with random sequencing errors, raw sequences were trimmed by eliminating reads that contained one or more ambiguous bases, had errors in the barcode or primer sequence, were

atypically short (70 bp), and had an average quality score <30 [27]. This process reduced the size of the whole dataset by ca 5%. To assess most abundant members of the microbiota, phylogenetic identity of generated sequences from bivalve samples were analysed using the CLC Microbial Genomics Module (v.9.5.1). Trimmed reads were first clustered at 97% similarity resulting in a total of 3,476 operational taxonomical units (OTUs). Singletons (OTUs with a single read in the data set) were excluded from the analysis. OTUs sequences were BLASTed against SILVA reference database of nearly 227,000 high quality rDNA genes for the prokaryotic kingdoms as described in the method session. The results of BLASTN were used to estimate the taxonomic content of the data set, using SILVA taxonomy with the CLC software. Only reads occurring at least five times in the trimmed data set were assigned to bacterial taxa and included in the results. Following these steps classification against SILVA reference database assigned 233,998 reads to the domain Bacteria while 65,982 remained unassigned.

Comparison of *C. gigas* and *M. galloprovincialis* Microbiota

In general, the composition of the bacterial community in both hemolymph and digestive gland of *C. gigas* and *M. galloprovincialis* was dominated by few OTUs accounting for the majority of reads in the analyzed samples (e.g., on average, the three most abundant OTUs, excluding non-classified phylotypes, accounted for >40% of total bacterial diversity).

Alpha-diversity metrics (e.g., number of total OTUs and bias-corrected form of Chao1 index) indicated that bacterial diversity and richness were higher in the haemolymph compared to the digestive gland in both bivalve species (Fig. 1). Although rarefaction curves computed for total OTUs abundance failed to reach a plateau (indicating that more sequencing effort should be required to detect additional phylotypes), rarefaction curves calculated for Chao1 index and other 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 (Fig. 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) (Fig. 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%) (Fig. 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 (Fig. 2). An additional large fraction of bacterial communities was represented by unclassified OTU sequences representing on average $20.1 \pm 6.5\%$ of the total bacteria diversity (Fig. 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 (Fig. 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) (Fig. 2).

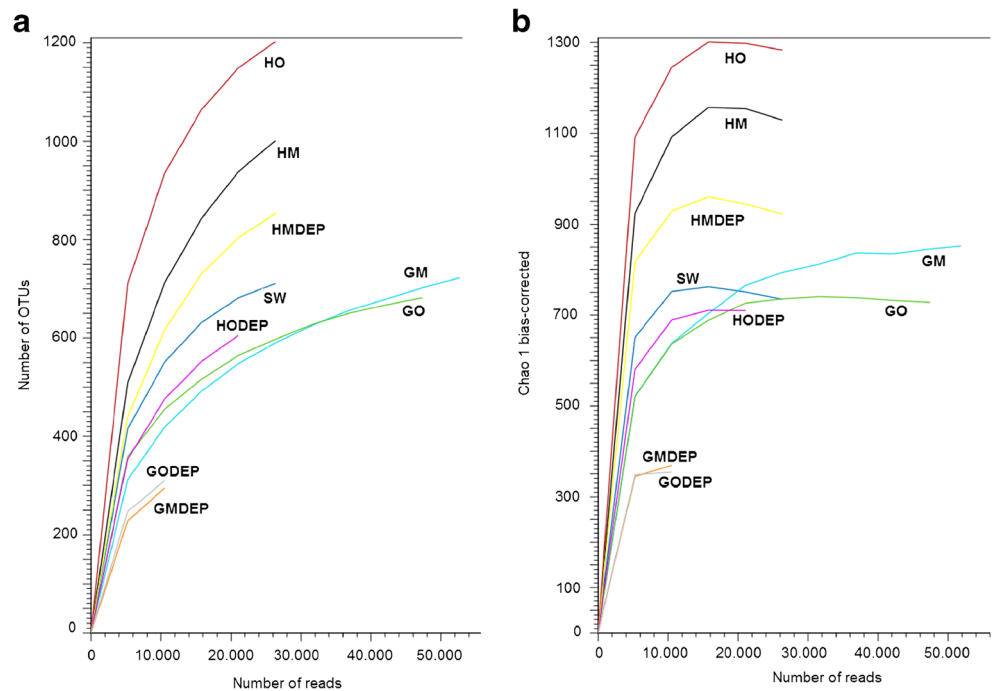
Distinct bacterial communities in seawater and bivalve tissues was also evident from the analysis of beta-diversity (Fig. 3). Beta-diversity analysis also showed that the composition of the bacterial community was very similar in the haemolymph of *C. gigas* and *M. galloprovincialis*, whereas differences were observed in the bacterial community composition of digestive gland in the two bivalve species (Fig. 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 (Fig. 1). In addition, both hemolymph and digestive gland samples from depurated bivalves clustered together in PCoA analysis, indicating a high level of similarity of bacterial diversity in these samples that differed significantly from beta-diversity metrics observed for non-depurated samples (Fig. 3).

A major shift in bivalve associated bacterial community structure following depuration practices regarded *Vibrio* populations that were found to significantly increase both in *C. gigas* and *M. galloprovincialis* tissues, with the only exception of *C. gigas* digestive gland (Fig. 2). In the hemolymph, the relative abundance of these bacteria increased from 24% to 42% in *C. gigas* and from 14 to 56% in *M. galloprovincialis*. A significant increase in the relative contribution of *Vibrio* to the whole bivalve bacterial community was also observed in the digestive

Fig. 1 Rarefaction curves for alpha-diversity metrics (number of total OTUs (a) and bias-corrected form of Chao1 index (b)) calculated from 16rDNA gene-based profiling analysis of the bacterial community in seawater and bivalve tissues (SW = seawater, HM = *M. galloprovincialis* haemolymph, HO = *C. gigas* haemolymph, GM = *M. galloprovincialis* digestive gland, GO = *C. gigas* digestive gland, DEP = deperated samples)



gland of *M. galloprovincialis*, where this bacteria increased from 29 to 54% following deperation.

Pseudoalteromonas, the dominant bacterial genus in the hemolymph of the two bivalve species was found to significantly decrease in *M. galloprovincialis* following deperation (relative abundance in the haemolymph from 25 to 9% before and after deperation, respectively) while it remained fairly constant in *C. gigas* samples (relative abundance in the haemolymph from 24 to 29% before and after deperation, respectively) (Fig. 2). Other major effects of deperation observed on bivalve microbiota were linked to a significant increase in the relative abundance of the genus *Stenotrophomonas* in the digestive gland of both *C. gigas* (relative abundance from <1 to 23% before and after deperation, respectively) and *M. galloprovincialis* (relative abundance from <1 to 26% before and after deperation, respectively) and to the disappearance of *Desulfovibrio* (relative abundance from 43 to 0% before and after deperation, respectively) in *M. galloprovincialis* digestive gland (Fig. 2).

Indigenous Potential Pathogenic *Vibrio* Species in *C. gigas* and *M. galloprovincialis* Tissues

Considering that the *Vibrio* genus represents a dominant group in bivalve tissues and play a role in bivalve disease, the presence of potential pathogenic *Vibrio* species, was investigated by real-time PCR in all samples (Table 1). The presence of *Vibrio* species pathogenic for humans and the fecal indicator *E. coli* were also assessed (Table 1).

The bivalve pathogen *V. aestuarianus* was found in oyster haemolymph, but not in oyster digestive gland and in

M. galloprovincialis samples. *V. coralliilyticus*, another potential bivalve pathogen, albeit found in seawater, was not detected in bivalve tissues. In contrast, bacteria belonging to the *Vibrio splendidus* clade, which includes a number of potential pathogenic species for bivalves (e.g., *Vibrio splendidus* and *Vibrio crassostrea*), were consistently found in *C. gigas* and *M. galloprovincialis* tissues at concentration ranging from 9.2×10^3 to 6.4×10^4 genomic unit (GU)/g (Table 1). Human pathogenic vibrio species *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were never detected in bivalve samples.

Deperation practices did not reduce the concentration of indigenous pathogenic vibrios (Table 1). In contrast, *V. aestuarianus* concentration increased from 2.4×10^2 cell/mL to 6.6×10^2 GU/g in the haemolymph of *C. gigas* following deperation. Likewise, concentration of bacteria belonging to the *V. splendidus* clade increased up to 3-fold in deperated samples as compared with non-deperated ones. The indicator bacteria *E. coli* used as control of the efficiency of deperation was only found in non-deperated bivalves (6.3×10^2 GU/g and 8.7×10^2 GU/g in digestive gland of *C. gigas* and *M. galloprovincialis*, respectively).

Discussion

Comparison of *C. gigas* and *M. galloprovincialis* Microbiota

In this work, for the first time to our knowledge, the microbiota structure in haemolymph and digestive gland of two contrasting bivalve species (*C. gigas* and *M. galloprovincialis*)

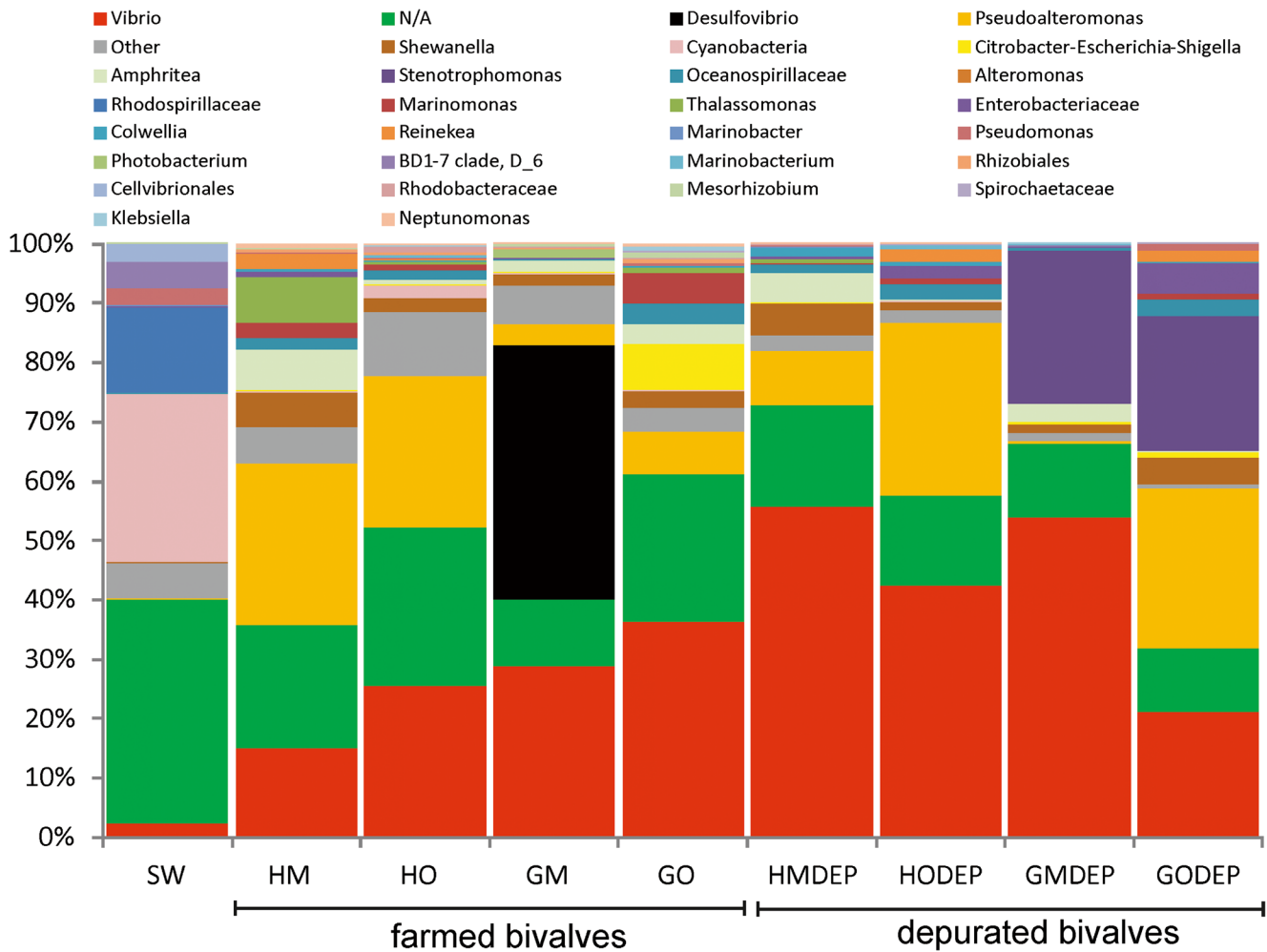


Fig. 2 Relative abundances of bacterial genera found in seawater and bivalve tissues by 16rDNA gene- based profiling analysis (SW = seawater, HM = *M. galloprovincialis* haemolymph,

HO = *C. gigas* haemolymph, GM = *M. galloprovincialis* digestive gland, GO = *C. gigas* digestive gland, DEP = depurated samples)

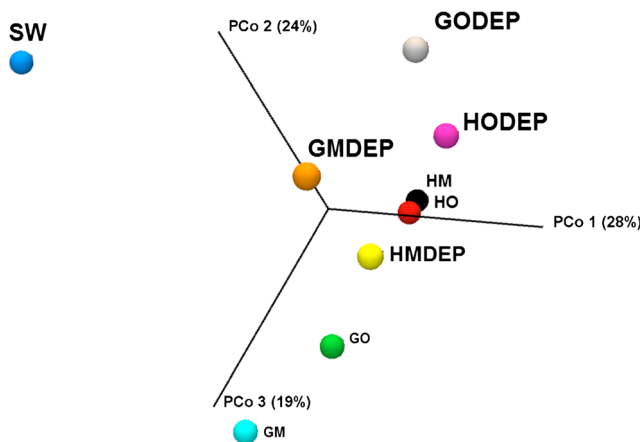


Fig. 3 3D PCoA plots of beta diversity for seawater and bivalve samples calculated using Principal Coordinate Analysis (PCoA) applied on Bray-Curtis distance matrix (SW = seawater, HM = *M. galloprovincialis* haemolymph, HO = *C. gigas* haemolymph, GM = *M. galloprovincialis* digestive gland, GO = *C. gigas* digestive gland, DEP = depurated samples)

that show different susceptibility to microbial diseases was evaluated by 16SrDNA gene amplification and pyrosequencing. Such comparison was made possible by taking advantage of co-cultivation of oysters and mussels in a Mediterranean fish farm where both species are grown under the same environmental conditions. The study was carried out during the warmest month of the year (August, SST 26.7 °C at the time of sampling), when susceptibility to diseases by bivalve species is generally highest.

Microbial communities associated with bivalves were significantly different from those of seawater, indicating the existence of a host-specific microbiota in bivalve tissues. Surprisingly, the microbiota associated to the haemolymph of the two bivalve species showed a high degree of similarity and it was dominated by the genera *Vibrio* and *Pseudoalteromonas*, both accounting on average for more than a third of the total bacterial community. These genera were reported in previous studies to represent dominant members of oyster microbiota [8, 28–30]. This not only indicates that these genera are able to persist in the

Table 1 Real-Time PCR counts of faecal indicators (*E. coli*), bivalve (*V. aestuarianus*, *V. splendidus*-clade, *V. coralliilyticus*) and human (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) potential pathogenic bacteria in seawater and bivalve tissues (SW = seawater,HM = *M. galloprovincialis* haemolymph, HO = *C. gigas* haemolymph, GM = *M. galloprovincialis* digestive gland, GO = *C. gigas* digestive gland, DEP = depurated samples)(GU = Genomic unit)

Sample	<i>E. coli</i> (GU/ml)	<i>V.aestuarianus</i> (GU/ml)	<i>V.splendidus</i> -clade (GU/ml)	<i>V.coralliilyticus</i> (GU/ml)	<i>V.cholerae</i> (GU/ml)	<i>V.parahaemolyticus</i> (GU/ml)	<i>V.vulnificus</i> (GU/ml)
SW	not found	not found	1.5×10^3	4.3×10^2	not found	not found	not found
	<i>E. coli</i> (GU/g)	<i>V.aestuarianus</i> (GU/g)	<i>V.splendidus</i> -clade (GU/g)	<i>V.coralliilyticus</i> (GU/g)	<i>V.cholerae</i> (GU/g)	<i>V.parahaemolyticus</i> (GU/g)	<i>V.vulnificus</i> (GU/g)
HM	not found	not found	4.2×10^4	not found	not found	not found	not found
HO	not found	2.4×10^2	1.5×10^4	not found	not found	not found	not found
GM	8.7×10^2	not found	1.9×10^4	not found	not found	not found	not found
GO	6.3×10^2	not found	not found	not found	not found	not found	not found
HMDEP	not found	not found	6.4×10^4	not found	not found	not found	not found
HODEP	not found	6.6×10^2	4.3×10^4	not found	not found	not found	not found
GMDEP	not found	not found	3.2×10^4	not found	not found	not found	not found
GODEP	not found	not found	9.2×10^3	not found	not found	not found	not found

haemolymph environment, and therefore to survive the antibacterial activity of hemolymph components, but also suggests that they might also contribute to bivalve antimicrobial defence for example through the production of antimicrobial compounds. Accordingly, *Pseudoalteromonas* species are generally found in association with marine eukaryotes and display anti-bacterial, bacteriolytic, agarolytic, and algicidal activities [31]. Vibrios are also commonly found in association with aquatic plants and animals, to which they may provide a chemical defence for the host [32]. However, the *Vibrio* genus also include bivalve pathogenic species, some of which are known to be associated with abnormal mortality outbreaks in farmed animals (e.g. *V. aestuarianus* and *V. splendidus* clade in farmed oysters). Interestingly, vibrios accounted for a larger fraction of the haemolymph microbiota in *C. gigas* compared to *M. galloprovincialis* (on average 1.7-fold), and the pathogen *V. aestuarianus* was detected by PCR in the hemolymph of *C. gigas*, but not in that of *M. galloprovincialis* (Table 1). This is in line with the lower susceptibility of mussels, with respect to oysters, to pathogenic *Vibrio* species also in relation to the presence in mussels of specific hemolymph soluble components (MgEP) that mediate adhesion, internalization and killing of *V. aestuarianus* by the hemocytes [18, 19, 33]. Overall, these results indicate that *Vibrio* might play a contrasting role in health and disease of different bivalve species.

In contrast to hemolymph, microbial communities in digestive gland displayed a more diversified composition in the two bivalves (Fig. 2). *Vibrio* still represented a significant fraction of the microbiota, whilst bacteria belonging to the *Pseudoalteromonas* genus were far less abundant in this tissue. As found in the haemolymph, *Vibrio* contribution to the overall microbial community structure was greater in *C. gigas* than in *M. galloprovincialis*, further supporting the hypothesis that the oyster environment may provide better conditions for

survival of these bacteria. From a functional perspective, the bivalve digestive gland plays a key role in the intracellular and extracellular digestion and storage of nutrients [34], and bacteria associated with this tissue may also contribute to this role. In this light, *Desulfovibrio* found in association with the mussel digestive gland is of particular interest, as bacteria belonging to this genus are capable to conduct sulphate respiration under strict anaerobic conditions [35]. Sulphate-reducing bacteria (SRB) are widespread in anaerobic environments, including the gastrointestinal tract of humans and other animals [35] (anaerobic conditions may establish in bivalve grow-out bags especially when bivalve are cultivated at high density as in the case of mussels). A potential role of these bacteria in anaerobic energy metabolism of *M. galloprovincialis* might thus be envisaged. In particular, they may contribute to the well-known capability of mussels to tolerate extended periods of hypoxia and anoxia, which may be induced by shell valve closure during emersion, depletion of oxygen in the surrounding water and other stressful conditions [36]. However, this remains a speculation and studies are in progress to confirm such observations and evaluate the role of SRB in mussel metabolism. It is finally worth noting that an additional large fraction of the hemolymph and digestive gland microbiota in the two bivalves is represented by unidentified microorganisms and rare taxa the role of which is yet to be assessed.

Effect of Depuration on *C. gigas* and *M. galloprovincialis* Microbiota

Depuration is a process by which bivalves are held in tanks of clean seawater under conditions which maximize the natural filtering activity enabling the removal of microbial and other contaminants before commercialization [37].

This process is used after collection and prior to commercialization of farmed bivalves resulting in a major decrease of coliforms and other transient bacteria in bivalve tissues. Albeit it is generally accepted that depuration practices are effective in removing allochthonous bacteria (e.g., enteric microorganisms) they are generally considered only partly effective, or ineffective, in removing other microbial contaminants such as naturally occurring marine vibrios [37].

Accordingly, the results of Real time-PCR indicate that *E. coli*, generally utilized as a marker of faecal contamination [38, 39], was completely removed from mussel and oyster tissues following depuration (Table 1). In contrast, indigenous *Vibrio* species (e.g., *Vibrio aestuarianus* and *V. splendidus* clade) were unaffected by the depuration treatment (Table 1). Although the existence of a host-specific microbiota in bivalves is still questioned, *Vibrio* species were found to represent a dominant fraction of both *C. gigas* and *M. galloprovincialis* microbiota (see previous paragraph). This observation suggests that these bacteria probably have a long co-evolutionary history with their invertebrate hosts, probably representing permanent residents of the microbiota, which in turn may lead to an increased resistance (persistence) to depuration. Interestingly, bacteria belonging to the *Vibrio* genus not only were unaffected by depuration practice but also tend to increase in abundance in depurated oysters and mussels when compared to non-depurated ones. Such findings might be better interpreted if we consider the more general influence that depuration had on the bivalve microbiota structure. In fact, the most prominent effect of depuration was a significant loss of microbial diversity in all samples from both bivalve species (Fig. 1). Phylotype loss was particularly evident for rare microbial taxa and, in turn, lead to a re-organization of dominant bacterial genera within the bivalve microbiota (Fig. 2). The increased dominance of bacteria belonging to the *Vibrio* genus within the overall bacterial community associated to both haemolymph and digestive gland of oysters and mussels may be probably related to new environmental niches that are made available through microbiota re-organization following depuration. Such an event is in line with the opportunistic nature of these bacteria, as also observed in other marine invertebrates for *Vibrio* pathogenic species during infection. For example, alteration of the coral microbiome under environmental stressful conditions, leading to decrease in dominant holobiont members such as the genus *Endozoicomonas*, has been linked to coral susceptibility to disease and infection by the coral pathogen *Vibrio coralliilyticus* [6, 40]. Similarly to vibrios, other bacterial groups such as the genus *Stenotrophomonas*, previously observed in association with oysters [41], 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 [42] have been proposed. However, none of these practices has yet been tested and proved to be successful in real aquaculture depuration facilities.

Conclusions

The results 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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