Tissue-scale microbiota of the Mediterranean mussel (*Mytilus galloprovincialis*) and its relationship with the environment

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HIGHLIGHTS

- *M. galloprovincialis* possesses microbiota with specific declinations at the tissue level.
- The digestive gland microbiota is enriched in fibrolytic SCFA producers.
- Gill and hemolymph microbiota are dominated by aerobic marine microorganisms.
- By releasing gill microorganisms, mussel farms affect the surrounding water ecosystems.
- *M. galloprovincialis* microbiota play a role in different aspects of host physiology.

GRAPHICAL ABSTRACT
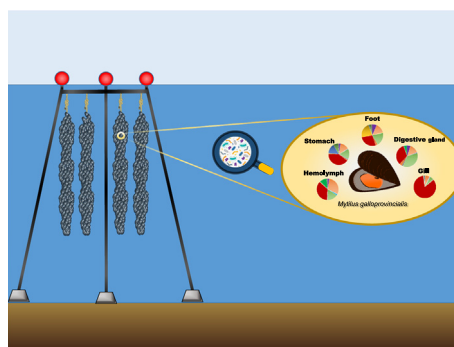
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ABSTRACT

In this study, we characterize the structural variation of the microbiota of *Mytilus galloprovincialis* at the tissue scale, also exploring the connection with the microbial ecosystem of the surrounding water. Mussels were sampled within a farm located in the North-Western Adriatic Sea and microbiota composition was analyzed in gills, hemolymph, digestive glands, stomach and foot by Next Generation Sequencing marker gene approach. Mussels showed a distinctive microbiota structure, with specific declinations at the tissue level. Indeed, each tissue is characterized by a distinct pattern of dominant families, reflecting a peculiar adaptation to the respective tissue niche. For instance, the microbiota of the digestive gland is characterized by *Ruminococcaceae* and *Lachnospiraceae*, being shaped to ferment complex polysaccharides of dietary origin into short-chain fatty acids, well matching the general asset of the animal gut microbiota. Conversely, the gill and hemolymph ecosystems are dominated by marine microorganisms with aerobic oxidative metabolism, consistent with the role played by these tissues as an interface with the external environment. Our findings highlight the putative importance of mussel microbiota for different aspects of host physiology, with ultimate repercussions on mussel health and productivity.

1. Introduction

Mediterranean mussels (*Mytilus galloprovincialis*) (Lamarck, 1819), like other bivalve mollusks, are key ecosystem engineers

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through their attachment to the substrate in dense mono- and multi-layered beds. Alongside their ecological importance, mussels have a relevant economic value as a species of interest in aquaculture and, at the same time, have long been employed in the biomonitoring of environmental quality in coastal areas (Faggio et al., 2018; Capolupo et al., 2017; Carella et al., 2018; Moschino et al., 2016). Indeed, their powerful filter feeding activity links them to the surrounding environment, allowing to filter large volumes of water, while concentrating different types of waterborne or particulate pollutants as well as microorganisms (Gagné et al., 2019; Pagano et al., 2016; Neori et al., 2004).

Up to now, most of the microbiological studies on mussels focused on the identification of pathogenic bacteria with deleterious health effects (Erol et al., 2016; Richards et al., 2010; Daczkowska-Kozon et al., 2010). However, marine organisms, including mussels, can be described as holobionts, given their life-long association with host-selected symbiont microbial communities known as microbiota (Pita et al., 2018; Glasl et al., 2016). These symbionts can endow the host with a set of probiotic functions (i.e. defense against pathogens, immunological regulation and improved nutritional efficiency), supporting homeostasis and health (O'Brien et al., 2019; Rausch et al., 2019; Simon et al., 2019). Consequently, the mussel microbiota should be considered as an integral component of the host physiology. However, to the best of our knowledge, only few and fragmentary studies aimed at the description of the mussel microbiota have been performed (Li et al., 2019; Vezzulli et al., 2018; Cappello et al., 2015; Kamada et al., 2013) and the resulting knowledge is still fragmentary.

The North-Western Adriatic Sea is characterized by a combination of shallow waters, restricted circulation and high riverine inputs (mainly through the Po river outflow) (Marini et al., 2008). These features affect its coastal areas that result as one of the most eutrophic environment and most productive area in the Mediterranean. Indeed, such conditions promote intense mussel farming, which is the prominent aquaculture activity in the area (Minarelli et al., 2018; Brigolin et al., 2017). Based on these considerations, mussel farms located in the North-Western Adriatic Sea are excellent field laboratories to explore the connection between the health and productivity of farmed mussels and the environmental quality. In an attempt to shed some light on the mussel microbiota structure and ecophysiology, here we applied a Next-Generation Sequencing (NGS) marker gene approach for the characterization of the symbiont microbial ecosystems of *M. galloprovincialis* collected from a mussel farm in the North-Western Adriatic Sea (Cesenatico, Italy). In particular, since recent studies carried out on the Manila clam (*Ruditapes philippinarum*) and the Pacific oyster (*Crassostrea gigas*) highlighted the existence of bivalve tissue-specific microbiota (Pathirana et al., 2019; Meisterhans et al., 2016; Lokmer et al., 2016), we explored the putative variation of *M. galloprovincialis* microbiota at the tissue scale. Hemolymph, gills, stomach and digestive gland are all important biological barriers between the animal and the environment, as well as sites for immunity, metabolism and detoxification (Freitas et al., 2019; Pagano et al., 2017; Franzellitti et al., 2016; Izagirre and Marigomez, 2009); hence, they host key functions for the mussel physiology. Thus, the dissection of microbiome specific variations at these tissues can provide a comprehensive vision of the putative role of microbiota in the host physiology. Seawater samples collected around the mussel farm and 3 miles away were also analyzed for microbiota composition. By characterizing the symbiont microbiota of *M. galloprovincialis* at the tissue scale and its connection with the microbiota of surrounding water, we aim at providing the basic knowledge for further, applied studies, with the purpose of unravelling the role of microbiota in bivalve health and productivity, possibly in relation to anthropic pressure and environmental pollution.

2. Materials and methods

2.1. Sampling and sample preparation

Mussel (*M. galloprovincialis* Lam.) sampling was carried out in April 2019 (spring season) in a farm located in Cesenatico, Italy (position: 44°09′04″N 12°32′60″E), by professional fishermen of the "Cooperativa Promoittica" (Cesenatico, Italy). The location is approved for direct commercialization of mussels (European legislation 91–492-EEC) and it is sited within an area routinely monitored by the Regional Agency for Prevention, Environment and Energy of Emilia-Romagna, Italy (ARPA-ER) to assess the status of the marine ecosystem and seawater quality (https://www.arpae.it). Twenty-five mussels of commercial size (5–7 cm in length) were collected and immediately stored in coolers (+4 °C) to be transferred within a few hours to the laboratory. In the laboratory, the mussels were cleaned and gently washed and then dissected under sterile conditions.

Specifically, for each animal, hemolymph was taken from the posterior adductor muscle using a sterile 1-ml syringe and transferred to a sterile tube. A 100-μl aliquot was employed to assess the health status of the animals through the evaluation of lysosomal membrane stability (LMS) on mussel hemocyte cells, according to Buratti et al. (2013). LMS was employed in these preliminary assessments as it is a proven sensitive and reliable biomarker of general health status in bivalves (Viarengo et al., 2007). The digestive gland, foot, gill and stomach were dissected from each individual as well, snap-frozen in liquid nitrogen, and stored at −80 °C along with hemolymph until analysis.

Two liters of seawater were collected at a depth of 3 m near the mussel farm (position: 44°9′04″N 12°32′60″E), as well as 3 miles away from the collection site (44°5′53″N 12°35′28″E) (Fig. S1). Seawater samples were stored in coolers (+4 °C) during transport to the laboratory and then immediately processed. A summary of the samples, sample size and handling methods is reported in Table S1.

2.2. Microbial DNA extraction

Total microbial DNA was extracted from approximately 20–30 mg of the digestive gland, foot, gill and stomach, and from 200 μl of hemolymph, using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with only minor adjustments in the homogenization step. Specifically, all samples were homogenized using the FastPrep instrument (MP Biomedicals, Irvine, CA) at 6 movements per s for 1 min. The elution step was repeated twice in 50 μl, incubating the columns for 5 min at 4 °C before centrifugation. DNA samples were stored at −20 °C for subsequent processing.

Seawater samples were filtered on 0.45-μm pore size MF-Millipore membrane filters using a vacuum pump. Total microbial DNA was extracted from membrane filters using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's protocol.

2.3. PCR amplification and sequencing

The V3–V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the 341F and 785R primers with added Illumina adapter overhang sequences, as previously described in Barone et al., 2019. The PCR program used was as follows: 95 °C for 3 min as initial denaturation, then 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and 5 min at 72 °C for the final elongation. PCR reactions were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR, using the Nextera technology (Illumina, San Diego, CA). After a further clean up step as described above, libraries were normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the

manufacturer's instructions. Sequencing reads were deposited in SRA-NCBI (project number PRJNA604916, mussel samples from SAMN14002069 to SAMN14002182, seawater samples from SAMN14002225 to SAMN14002231).

2.4. Bioinformatics and statistics

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019; https://qiime2.org). High-quality reads were clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using the VSEARCH classifier (Rognes et al., 2016) and the SILVA database as a reference (Quast et al., 2013). Unassigned sequences and those assigned to eukaryotes (i.e. chloroplasts and mitochondrial ones) were discarded.

Alpha rarefaction was performed using Faith's Phylogenetic Diversity (PD whole tree). A trade-off rarefaction value of 1900 reads per sample was chosen to capture the extent of diversity in our data. Beta diversity was estimated by computing weighted and unweighted UniFrac distances.

All statistical analysis was performed using R version 3.5.1 (https://www.r-project.org/). Unweighted UniFrac distances were plotted using the vegan package, and the significance of data separation in the principal coordinates analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function adonis in the vegan package). Alpha diversity was evaluated using two different metrics: Simpson Index (complement) and observed ASVs. Between-tissue differences for alpha diversity were assessed by Wilcoxon test. P-values were adjusted for multiple comparisons using the false discovery rate (FDR) (function p.adjust in the stats package), and a P-value ≤ .05 was considered as statistically significant. Representative sequences of taxa of interest were aligned to the 16S Microbial NCBI database (release September 2019) with BLASTn (version 2.9.0), considering at least 80% of sequences identity. Metagenome prediction of SILVA-picked ASVs was performed with PICRUSt2 (Barbera et al., 2019; Czech and Stamatakis, 2019; Douglas et al., 2019; Louca and Doebeli, 2018; Ye and Doak, 2009), using Metacyc (Caspi et al., 2018) as reference for pathway annotation and a NSTI threshold of 2. Over- abundant pathways in the different mussel organs and seawater were obtained in pairwise Wald tests, as implemented in DESeq2 package (Love et al., 2014). Over-abundant pathways with Bonferroni corrected P-value ≤.05 and an absolute ($\log_2$ fold change) ≥ 2 were retained. Sample clustering was performed accordingly to the pathways abundance profiles, adopting Kendall's correlation coefficients as metric and Ward-linkage method.

3. Results

3.1. NGS-based profiling of M. galloprovincialis microbiota and the surrounding seawater

A total of 121 samples (25 digestive glands, 25 gills, 21 stomachs, 25 feet, 18 hemolymph samples and 7 seawater samples) were analyzed (Table S1). For each sample, the microbiota structure was profiled by NGS of the V3–V4 hypervariable region of the 16S rRNA gene. A total of 5,621,255 paired-end sequences passed quality filtering (mean per sample ± SD, 46456 ± 68,116). High-quality reads were clustered into 18,787 ASVs (8532 ± 4634).

The overall composition of the *M. galloprovincialis* microbiota is reported in Fig. 1A. The phyla Proteobacteria (mean relative abundance (r.a.) ± SD, 44.8% ± 27.2%), Firmicutes (18.5% ± 20.2%) and Bacteroidetes (14.8% ± 12.8%) dominated the ecosystem. Spirochaetes, Verrucomicrobia, Actinobacteria, Tenericutes, Planctomycetes, Cyanobacteria, Fusobacteria, Chloroflexi and Chlamydiae were subdominant components, with a mean r.a. of about 5%. At the family level, the most represented taxa were an unclassified family of the

Alteromonadales order (10.7 ± 21.6%) and *Flavobacteriaceae* (8.8% ± 9.6%) (Fig. 1B). *Spirochaetaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Bacillaceae*, *Vibrionaceae*, *Verrucomicrobiaceae*, *Hahellaceae* and *Rhodobacteraceae* were subdominant families, showing a mean r.a. ranging from 3% to 5%. Consistently, among the dominant genera we reported unclassified taxa of Alteromonadales (10.6% ± 21.4%) and *Flavobacteriaceae* (5.4% ± 6.3%). *Spirochaeta 2*, *Bacillus*, *Vibrio*, *Endozoicomonas*, an unclassified genus of *Verrucomicrobiaceae* and *Mycoplasma* were all subdominant genera with mean r.a. between 3% and 5% (Fig. 1C).

As for seawater, Proteobacteria (68.6 ± 8.4%) and Bacteroidetes (14.8% ± 3.2%) were the dominant phyla (Fig. 1A), with Actinobacteria, Verrucomicrobia and Planctomycetes being subdominant components (mean r.a., 5%). The most represented families were *Pseudoalteromonadaceae* (11.6% ± 8.6%), *Flavobacteriaceae* (11.0% ± 4.3%) *Vibrionaceae* (9.3% ± 9.2%), *Rhodobacteraceae* (8.8% ± 4.0%) and *Halieaceae* (6.1% ± 5.9%). *Microbacteriaceae*, *FamilyI* of Cyanobacteria, *Campylobacteraceae*, *Planctomycetaceae*, and *Verrucomicrobiaceae* were subdominant components, with a mean r.a. ranging from 2% to 5% (Fig. 1B). At the genus level, *Pseudoalteromonas* (11.7% ± 8.4%), *Vibrio* (9.0% ± 8.7%), and unknown genera belonging to the *Rhodobacteraceae* (7.5% ± 4.8%) and *Halieaceae* families (6% ± 6.6%) were the dominant taxa. Among the subdominant ones, *Synechococcus*, *Arcobacter* and an unclassified genus of *Verrucomicrobiaceae* were present, all showing average r.a. between 2% and 3% (Fig. 1C).

3.2. Tissue-specific composition of M. galloprovincialis microbial ecosystems

To explore peculiarities of microbiota composition in the different tissues of *M. galloprovincialis*, an unweighted UniFrac-based PCoA of the compositional profiles of mussel samples, as well as of seawater, was carried out. As expected, the seawater samples clustered apart from all mussel organs (Fig. 2A) and the mussel samples significantly segregated according to the tissue type (permutation test with pseudo-F ratios, P-value ≤.001). To assess the degree of microbiota variation between tissues, pairwise adonis permutation tests were performed (Table S2). Even if showing overall low R^2 values, all between-tissue comparisons of the microbiota structure were found to be significant (P-value ≤.03), highlighting the high level of organ specificity of mussel microbiota (Fig. 2A).

With regard to alpha diversity, no significant differences in species richness were found among the seawater and mussel ecosystems. However, the gill microbiota showed lower evenness (calculated as Simpson index- complement) than that of the digestive gland and stomach (Wilcoxon test, P-value <.03).

For what concerns the compositional structure, the microbiota from each organ showed a specific layout of dominant families (Fig. 3). In particular, *Ruminococcaceae* (mean r.a. ± SD, 14% ± 14%) and *Lachnospiraceae* (10% ± 13.2%) dominated the digestive gland microbial ecosystem. *Spirochaetaceae* were dominant in the foot (2% ± 26%), while an unclassified family of the Alteromonadales order (43% ± 25%) and *Hahellaceae* (11% ± 9.6%) dominated the gills, *Mycoplasmataceae* (15% ± 18%) the stomach and *Flavobacteriaceae* (19% ± 11.2%) the hemolymph (Fig. 3). The relative abundance of the most represented families in all Mediterranean mussel organs and seawater is provided in Table S3.

3.3. Impact of mussel farming on the microbiota composition of the surrounding seawater

Intending to assess the impact of mussel farming on the surrounding seawater, we compared the microbiota composition between 6 seawater samples collected close to the mussel farm and a sample collected 3 miles away from the farm as a control (Fig. S1).
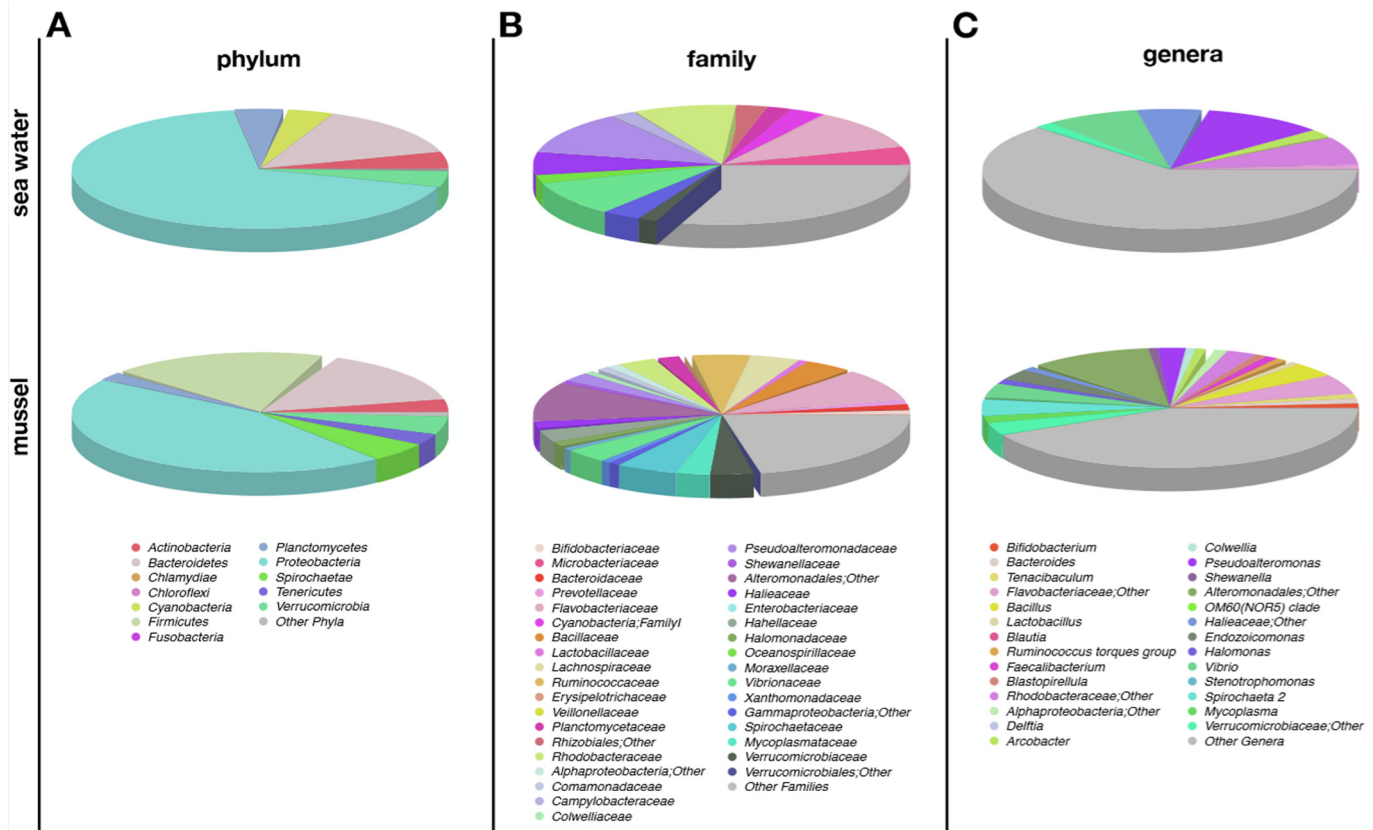
Fig. 1. The whole *Mytilus galloprovincialis* and seawater microbiota. Pie charts summarizing the phylum- (A), family- (B) and genus-level (C) microbiota composition of Mediterranean mussels and seawater. Only phyla with relative abundance ≥1% in at least 10% of samples, families with relative abundance ≥1.5% in at least 10% of samples and genera with relative abundance ≥2% in at least 10% of samples are represented.

As shown in Fig. S2, we noticed a variation in the family-level relative abundance profiles between the seawater surrounding the mussel farm and the control water. In particular, the families *Pseudoalteromonadaceae* and *Verrucomicrobiaceae* showed greater relative abundance in the seawater surrounding the mussel farm than in the control water (mean r.a. ± SD, 13.4% ± 7.9% vs. 1%, and 1.9% ± 1.7% vs. 0%, respectively), while *Halieaceae* was more represented in the control water (r.a., 10.9% vs. seawater near the farm, 5.3% ± 6.4%).

Interestingly, the family *Vibrionaceae*, which includes several species known as opportunistic and potential pathogens of marine organisms (Baker-Austin et al., 2018; Le Roux et al., 2015), was also more represented in the seawater surrounding the mussel farm (10.6% ± 9.3%) than in the control water (1.5%). In order to identify the *Vibrionaceae*-related ASVs down to species level, their sequences were mapped onto the 16S Microbial NCBI database. The best hit was *Vibrio splendidus* (>80% identity), a well-known potential pathogen.

3.4. Predicted functional profiling of M. galloprovincialis and seawater microbiomes.

To gain insight into the peculiar functional variations of the microbiota in the different *M. galloprovincialis* organs/tissues, as well as in the seawater, correspondent metagenomes were inferred from the phylogenetic profiles using PICRUSt2. A differential abundance analysis was carried out, resulting in 94 Metacyc pathways being significantly over-abundant in at least one mussel organ or seawater metagenome (Supplementary Table S4). Samples were then clustered according to the abundance profile of the 94 over-abundant pathways (Fig. 4). Even if a certain level of dispersions was maintained, samples showed an overall tendency towards the segregation between water, gills and hemolymph. A cluster including stomach, digestive glands and foot samples

was also obtained. The clustering analysis indicated for seawater a distinguished functional profile, characterized by the enrichment in pathways involved in nitrogen cycle (i.e. L-histidine degradation II and nitrate reduction VI) and in the degradation of the aromatic compound gallate. Although sharing several functionalities with the seawater microbiome, hemolymph was characterized by the over-abundance of pathways involved in sulfur metabolism (i.e. super-pathway of sulfolactate degradation), in the regulation of osmolarity (i.e. super-pathway of taurine degradation and glycine betaine degradation I) and in the degradation of aromatic compounds (i.e. protocatechuate degradation II). Conversely, gills microbiome showed an enrichment in pathways involved in the respiratory electron transport (i.e. quinol and quinone biosynthesis). Notably, the digestive gland and the stomach microbiomes were both characterized by pathways involved in fermentation (i.e. pyruvate fermentation to acetate and lactate II and heterolactic fermentation) and in the degradation of several aromatic compounds (i.e. catechol, nicotinate, salicylate and toluene).

4. Discussion

In the present study, we characterized the Mediterranean mussel microbiota, also exploring its structural variation at the tissue scale and the connection with the microbial ecosystem of the surrounding seawater. According to our findings, the mussel microbiota was well differentiated from that of seawater. Indeed, at the phylum level the mussel microbiota was dominated by Proteobacteria, Firmicutes and Bacteroidetes, while that of seawater showed only Proteobacteria and Bacteroidetes as dominant phyla. However, it is at lower phylogenetic levels that the differences between the animal and seawater ecosystems were more evident. While showing a similar pattern of dominant families, which mainly encompasses microorganisms of marine origin, such
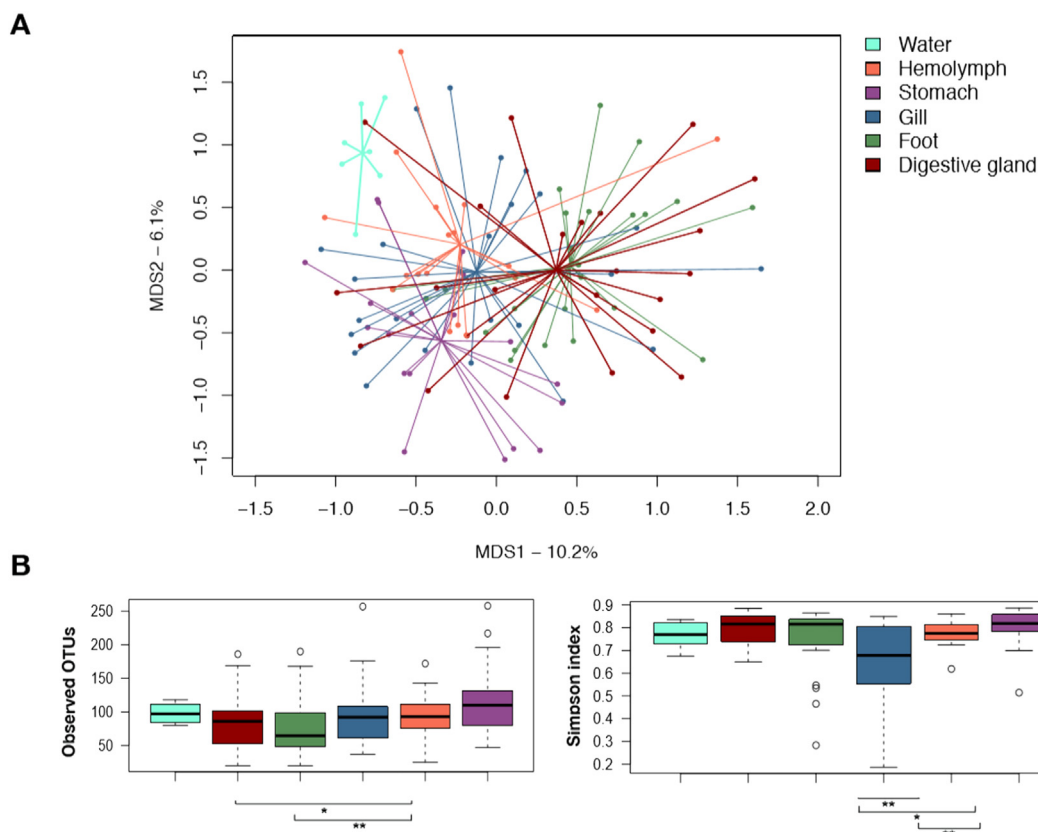
Fig. 2. Alpha and beta diversity of *M. galloprovincialis* tissue and seawater microbiota. (A) PCoA based on unweighted UniFrac distances between the microbiota structures of the samples taken from each organ of *M. galloprovincialis* and seawater. Samples are significantly separated (permutation test with pseudo-F ratios, P-value ≤.001). (B) Box and whiskers plots showing the alpha diversity values, measured as amplicon sequence variants (ASVs) and Simpson index- complement. *, P-value ≤.05, Wilcoxon test. The color legend is depicted at the top-right of the plot in panel A.

as *Flavobacteriaceae*, Alteromonadales and *Rhodobacteriaceae*, the subdominant fraction of the mussel and seawater ecosystems was remarkably different. Unlike seawater, which was characterized by a vast diversity of marine taxa, the mussel microbiota included well-known animal microbial commensals, such as *Ruminococcaceae*, *Lachnospiraceae* and *Bacillaceae*, and possibly opportunistic microorganism such as members of the family *Spirochaetaceae*. The inferred metagenomics also highlights an overall distinct functional profile between the seawater and the mussel microbiomes. While seawater was characterized by functions involved in sulfur and nitrogen cycling, the mussel ecosystems are enriched in genes involved in carbohydrates oxidation or fermentation, with specific variations depending on the tissue. Taken together, these data may indicate the propensity of mussels to select and retain microorganisms with animal tropism, such as symbiotic microbial partners. A similar behavior was recently reported for the sea cucumber, *Holothuria glaberrima* (Pagán-Jiménez et al., 2019), in which the presence of *Ruminococcaceae* and *Lachnospiraceae* in the gut was suggested to influence the gastrointestinal metabolism of the host, as well demonstrated in terrestrial animals.

To better dissect the possible contribution of the mussel microbiota to the host physiology, we explored its variation at the tissue scale. Interestingly, although showing an overall comparable biodiversity, the microbiota of each tissue was characterized by a specific pattern of dominant families, suggesting a peculiar ecological propensity. For instance, being dominated by *Ruminococcaceae* and *Lachnospiraceae*, the digestive gland microbiota was configured as an anaerobic ecosystem enriched in commensal microorganisms capable of fermenting complex polysaccharides to short-chain fatty acids (SCFAs), well matching the general asset of animal gut microbiota (Muegge et al., 2011). Indeed, the digestive gland is the main site for the digestive, metabolic, and detoxification functions of mussels. These physiological activities may

contribute to the establishment of suitable conditions to promote anaerobic bacteria capable of producing SCFAs through the fermentation of dietary fibers (Saltzman et al., 2017), such as cellulose and hemicellulose (La Reau et al., 2016), which are commonly found in bivalve food, as dinoflagellate algae (Arapov et al., 2010; Rouillon et al., 2005). Further supporting this considerations, a recent study showed that an α-D-glucan (MP-A) polysaccharide isolated from the mussel *Mytilus coruscus* affects the gut microbiota composition in Sprague Dawley rats fed with a high-fat diet, promoting SCFA production and alleviating the deleterious effects of the diet (Wu et al., 2019). Similarly, the stomach and foot microbiota were dominated by anaerobic microorganisms with animal tropism, especially *Spirochaetaceae* and *Mycoplasmataceae*. These microorganisms are generally considered as opportunistic rather than commensal, at least in mammalian hosts (Hampson and Ahmed, 2009; Waites and Talkington, 2004). However, according to van de Water et al. (2016), Spirochaetes members may act as symbionts in mollusks. Gills and hemolymph microbiota showed a completely different ecological structure compared to the other tissues, being dominated by aerobes of marine origin, such as Alteromonadales and *Hahellaceae* (gill), and *Flavobacteriaceae* (hemolymph). These findings well agree with the common role of the tissues as a primary biological barrier between the animal and the external environment, being in direct contact with the surrounding seawater. Therefore, their microbial composition most likely reflects the conditions imposed by the external environment, as observed in previous studies (Brito et al., 2018). On the other hand, gills and hemolymph may also exert an active role in the selection of microbial symbionts composing the microbiota of internal tissues (i.e. digestive gland and stomach), by means of filtering activity (gill), or immune recognition and phagocytosis operated by hemocytes and translocation to other organs/animal districts (hemolymph) (Ikuta et al., 2019; Burgos-Aceves and Faggio, 2017). Inferred metagenomes at the
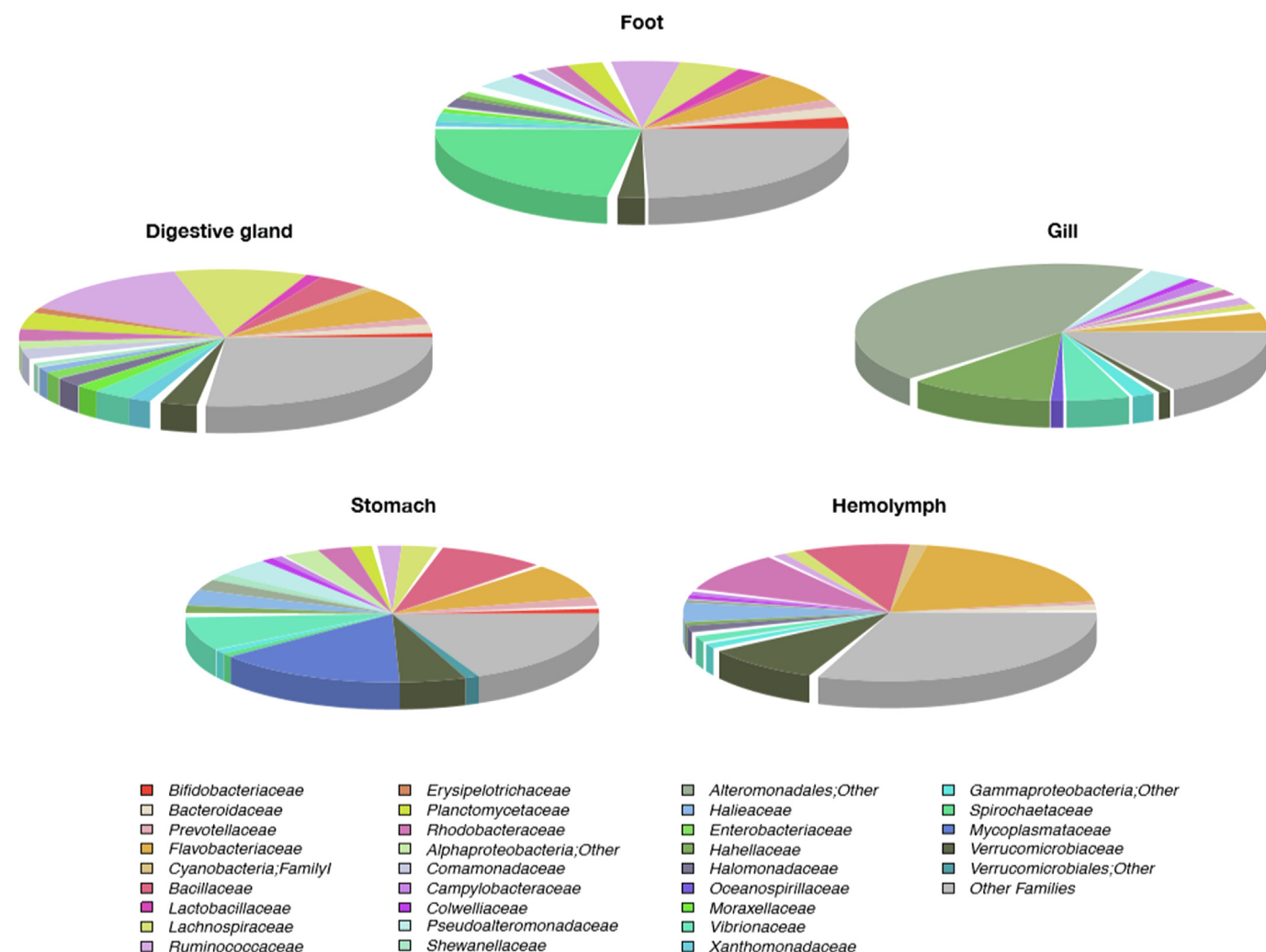
Fig. 3. The tissue-specific *M. galloprovincialis* microbiota composition at the family level. Pie charts summarizing the family-level microbiota composition of the digestive gland, foot, gill, stomach and hemolymph of *M. galloprovincialis*. Only bacterial families with a relative abundance of ≥1.5% in at least 10% of samples are represented.

tissue scale confirmed the hypothesized metabolic propensities of the corresponding microbiota. Indeed, according to our findings, the gills ecosystem was characterized by functions involved in oxidative respiration, while the stomach and, particularly, the digestive gland, were over-abundant in functions related to the fermentation of polysaccharides and the degradation of aromatic compounds. These specificities of mussel microbiota at the tissue scale were robust to inter-individual variability. This suggests that the main determinant of the mussel microbiota variation is the niche-specificity rather than the individual differences. The same behavior was observed for mammals, where the structure of symbiont microbial ecosystems segregates according to the body district (Integrative HMP-iHMP- Research Network Consortium, 2014).

Finally, we explored the impact of mussel farming on the microbiota of the surrounding water. Compared to the control seawater (i.e. water collected 3 miles away from the mussel farm), seawater collected close to the farm was enriched in *Pseudoalteromonadaceae*, *Verrucomicrobiaceae* and *Vibrionaceae*, while being depleted in *Halieaceae*. This data emphasizes the potential of mussel farming to directly affect microbial ecology of seawater by releasing microorganisms that characterize the gill (i.e. *Vibrionaceae* and *Pseudoalteromonadaceae*) and hemolymph (i.e. *Verrucomicrobiaceae*), while retaining *Halieaceae* in the mussel microbiota. These findings further stress the close contact between gill/hemolymph and the external environment, as well as the function displayed by both tissues as the main route for tissue uptake

of waterborne compounds and particulate material (including microorganisms).

5. Conclusions

Our study provides the first integrative description of the mussel microbiota variation at the tissue scale. According to our findings, mussels possess a characteristic microbiota, well differentiated from the seawater micro-ecosystem, with robust compositional variations at the organ level. Indeed, while gill and hemolymph ecosystems are generally dominated by aerobic marine microorganisms, foot, stomach and digestive gland microbiota are characterized by anaerobes with tropism for animal tissues. In particular, being dominated by *Ruminococcaceae* and *Lachnospiraceae*, the microbiota of the digestive gland appears to be well structured for the production of SCFAs from complex polysaccharides. As health-promoting bioactive small molecules (Turroni et al., 2017), SCFAs may be effective in modulating the host metabolic and immunological layout (Hu et al., 2018; Ikeda-Ohtsubo et al., 2018), providing the mussel holobiont with important probiotic functions, including immune stimulation and cell signaling. Our findings promote further research to better understand the role of mussel microbiota in different aspects of host physiology. In particular, by means of shotgun metagenomics more information could be provided on the mussel microbiome structure also in response to environmental and anthropic stressors, to highlight the ultimate impact on health and productivity.
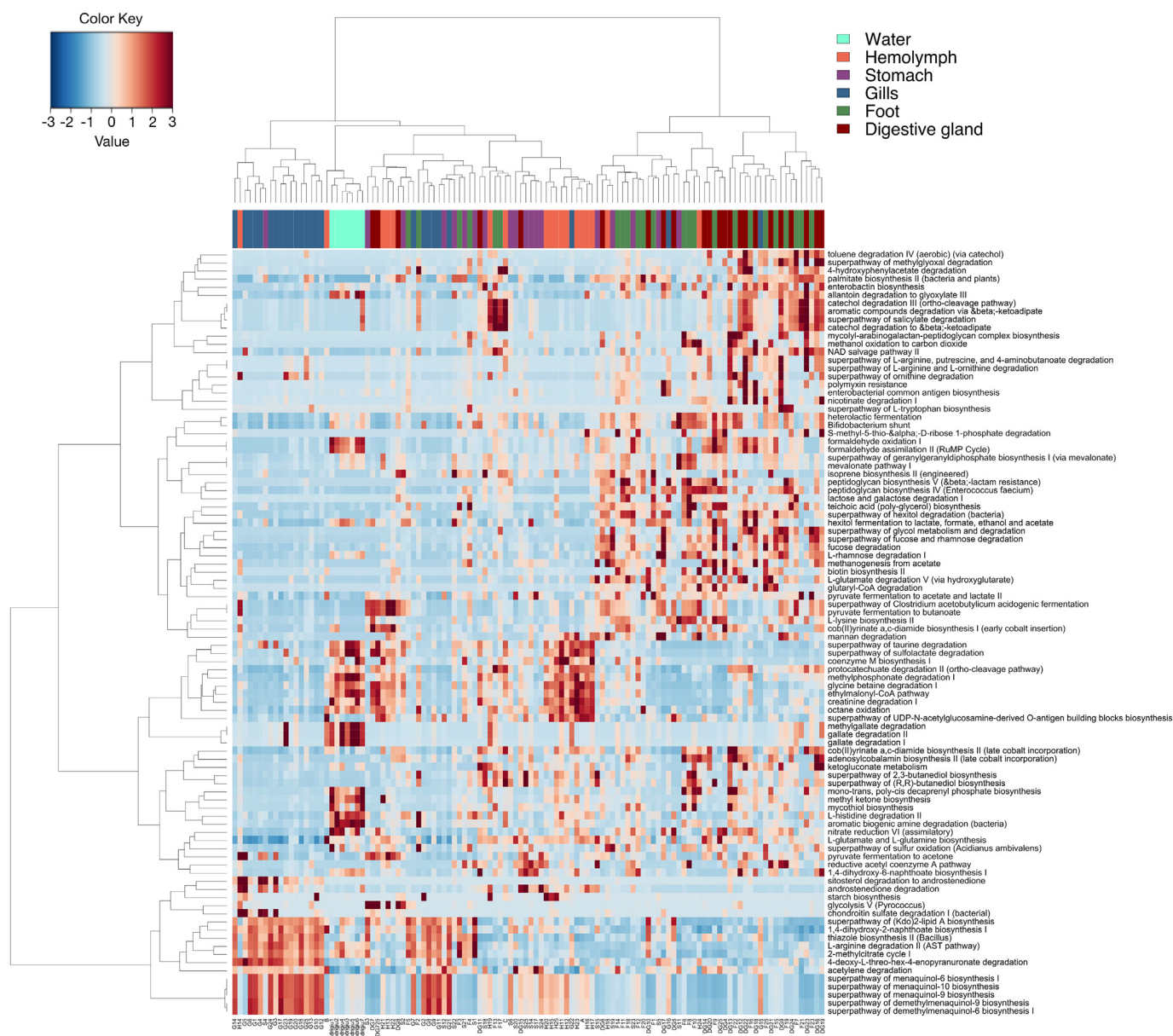
Fig. 4. Hierarchical clustering of the inferred metagenomes from the different tissue of *M. galloprovincialis* and seawater. The heatmap shows Ward-linkage clustering based on the Kendall correlation coefficients of the sample abundances profile of the 94 over-abundant pathways (Wald test logarithmic fold change of 2, P-value ≤.05). Samples are shown column-wise and colored by tissues. Metabolic pathways, named from the Metacyc database, are reported on the rows.

Likewise, the temporal dynamics of mussel microbiota in the different organs need to be described, to dissect the key time windows and developmental stages for the microbiota establishment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137209.

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