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Organ-specific bacterial communities of the soft-shell clam *Mya arenaria* (Linnaeus, 1758) and adjacent sediments in the Black Sea

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Bacteria colonizing bivalves play a critical role in host health by supporting digestion, nutrient cycling, and immune defense. While the microbiomes of marine bivalves have been studied globally, their diversity and functional roles across specific organs remain underexplored. This study investigates the structural and predicted functional diversity of bacterial communities associated with different organs (siphon, gills, and stomach) of the marine bivalve *Mya arenaria* Linnaeus, 1758, along with the surrounding sediments from the Romanian Black Sea coast, using 16S rRNA gene sequencing with Illumina technology. Bacterial communities within the bivalve differed markedly from those in the sediments and varied across organs. Sediment samples exhibited greater taxonomic diversity (19 phyla) than bivalve organs (14–15 phyla). Verrucomicrobiota dominated the siphon and gills, Spirochaetota were most abundant in the stomach, and Desulfobacterota predominated in sediments. Nitrate-reducing bacteria, particularly those from the genus *Persicirhabdus*, were prevalent in all organs and may contribute to host resilience under hypoxic conditions. The presence of *Sulfurimonas* in the stomach suggests a possible nutritional association, while halotolerant *Woeseia* species identified in sediments likely play a role in environmental nutrient cycling. Predictive functional profiling indicated potential bacterial involvement in various metabolic processes, including carbohydrate, amino acid, and energy metabolism. Additionally, pathways related to xenobiotic degradation and antibiotic biosynthesis were inferred across all sample types, indicating a potential capacity for broader ecological and possibly

biotechnological roles. However, these functions were inferred from 16S rRNA data and require further validation through metagenomic or transcriptomic approaches. To our knowledge, this is the first detailed analysis of microbiome variability across different organs of *M. arenaria*, offering new insights into host–microbe interactions in this species.

KEYWORDS

invertebrates' bacteria, *Mya arenaria* microbiome, Black Sea bivalve, Illumina sequencing, 16S rRNA gene

1 Introduction

Microorganisms are widespread and essential components in marine ecosystems, establishing diverse and often intimate associations with a wide range of animals (Fraune and Bosch, 2010; Krishnaveni et al., 2025). Marine benthic invertebrates, like all multicellular organisms, engage in complex interactions with a diverse array of microbes, including bacteria (Knowlton and Rohwer, 2003; Li et al., 2023a, b), and these associations can take the form of mutualism, commensalism, or parasitism. Bacteria colonizing marine invertebrates play crucial roles in maintaining host health, supplying essential nutrients, and offering protection against pathogens. Moreover, they contribute significantly to the overall functioning and adaptive responses of hosts to environmental change (Bourne et al., 2016; Pita et al., 2018). Hosts may possess species-specific bacterial communities distinct from their surrounding environment, which can be acquired vertically, from conspecifics, or from the environment (Koskella et al., 2017). The degree of specificity of these communities varies among host species, with some exhibiting only a few highly specific symbionts, whereas others share bacteria with the surrounding environment (Lemieux-Labonté et al., 2016; Lemay et al., 2018; Roth-Schulze et al., 2018).

Bivalves harbor a wide variety of microorganisms, including protozoa, fungi, viruses, and bacteria (Rey-Campos et al., 2022), with bacteria being the most extensively studied group (Masanja et al., 2023). The microbiome of bivalves plays a critical role in the host health and disease, with the bacterial component involved in processes such as digestion, nutrient cycling, and immune defense (Pierce and Ward, 2018; Timmins-Schiffman et al., 2021). Certain bivalve-colonizing bacteria have been found to produce antimicrobial compounds that help protect the host from pathogens (Destoumieux-Garzon et al., 2020; Balbi et al., 2021). In addition, some species belonging to the families *Phyllobacteriaceae* and *Hyphomicrobiaceae* (*Pseudomonadota*) and *Flavobacteriaceae* (*Bacteroidota*) are associated with enhanced disease resistance in bivalves (Dubé et al., 2019). These microorganisms produce enzymes that break down complex carbohydrates and contribute to the immune response of their host (Moruf et al., 2020).

The structure of microbial communities colonizing marine invertebrates, including bivalves, is highly variable, with different body regions harboring distinct microbial communities (Masanja et al., 2023). For instance, the bacterial composition of *Eriocheir sinensis* (Chinese crab) was dominated by *Tenericutes* and *Pseudomonadota* in the gut, whereas *Actinobacteria*, *Pseudomonadota*, and *Bacteroidetes* predominated in the gill samples (Zhang et al., 2016). Similarly, *Mytilus galloprovincialis* possesses a microbiota with tissue-specific variations: where the digestive gland is characterized by *Ruminococcaceae* and *Lachnospiraceae*, the stomach is dominated by *Flavobacteriaceae*, and the foot by *Spirochaetaceae* representatives (Musella et al., 2020).

The Boreal-Atlantic bivalve *Mya arenaria* Linnaeus, 1758, is native to the North Atlantic, off the coasts of America and Canada (Strasser, 1998). Invasive in Europe, it has been reported in the Barents Sea (Galkin, 1998), White (Russanova, 1963), Baltic (Kube, 1996), North (Strasser, 1998), Wadden (Smidt, 1951), Mediterranean seas (Stora et al., 1995), and along the European coasts of the Atlantic Ocean (Conde et al., 2011). In the Black Sea, it was first recorded in 1966 near Odessa (Beshevli and Kolyagin, 1967; Gomoiu and Porumb, 1969), later identified near Berezan Island (Zambriborshch et al., 1968), and within a relatively short time became a common species in the northwestern part of the basin. Considering the age of the identified specimens, as well as the time required for larval development and reaching sexual maturity, Zambriborshch et al. (1968) suggested that the species entered the Black Sea in the early 50s. In the Romanian Black Sea region, this species was first reported in the late 1960s, with a limited number of specimens identified near Zaton and Tomis Harbor (Constanța) (Gomoiu and Porumb, 1969), and was later observed on the Romanian continental shelf at depths of up to 35 m (Skolka and Gomoiu, 2004).

This bivalve thrives in the organic-rich muds of the Black Sea, particularly in sedimentary habitats influenced by inputs from the Danube River, which carry high bacterial loads, including potential pathogens (Ene et al., 2025). Although not currently consumed in Romania, *M. arenaria* is recognized for its edible potential and is successfully harvested and farmed in other regions of the world (Wheaton et al., 2008). In the Black Sea, it serves as prey for several

commercially important benthic fish species, potentially raising public health concerns. Despite exposure to bacterial pathogens, the species appears to remain unaffected, likely due to immune adaptations that enable tolerance to challenging environmental conditions such as hypoxia, rapid fluctuations and high organic pollution. Some studies have shown that the soft-shell clam hosts a distinct microbiome, suggesting a possible symbiotic relationship between the clam and its associated microorganisms (Cabelli and Heffernan, 1970; Liu et al., 2020).

While extensive research has been conducted on bacterial communities colonizing benthic organisms in marine environments (Gilbertson et al., 2012; Furst et al., 2021; Li et al., 2023b; Liu et al., 2023), including bivalves (Musella et al., 2020; Akter et al., 2023), only a few studies have addressed the microbiome of these invertebrates from the Black Sea, notably in the bivalves *Mytilaster lineatus* (Onishchenko and Kiprianova, 2006) and *Donax trunculus* (Ibryamova et al., 2022), as well as the tube-dwelling polychaete *Melinna palmata* (Menabit et al., 2024).

Microbial ecology studies carried out in the Black Sea ecosystem have predominantly focused on bacterial communities from seawater (Jørgensen et al., 1991; Sorokin et al., 1995; Glaubitz et al., 2010; Bryukhanov et al., 2015; Ruginescu et al., 2022) and sediments (Schulz et al., 1999; Thamdrup et al., 2000; Leloup et al., 2007; Schäfer et al., 2007; Coolen and Shtereva, 2009; Schippers et al., 2012), with limited comparison between their diversity and that of invertebrate-associated communities (Onishchenko and Kiprianova, 2006; Menabit et al., 2024).

In this context, the present study aims to assess the structural and functional bacterial diversity associated with the marine bivalve *M. arenaria*, focusing on organ-specific (siphon, gills, and stomach) microbiomes and their distinctions from surrounding sediment communities using a high-throughput Illumina 16S rRNA gene sequencing approach. The study was based on the hypothesis that *M. arenaria* harbors a microbiome that facilitates its adaptation to stressful estuarine environments and organic pollution, including bacterial contaminants. Rather than using classical microbiological methods, we applied molecular techniques that enabled the identification of bacterial genes potentially involved in diverse metabolic functions, including antibiotic resistance. This investigation represents the first characterization of microbiome variability across different organs of the bivalve *M. arenaria*.

2 Materials and methods

2.1 Study area and sample collection and preparation

The north-western Black Sea covers 127,000 km², accounting for 94% of the shelf's total area, and approximately 1.2% of the total Black Sea water volume. This region receives significant freshwater input from the Danube, Dniester, Dnieper, and Southern Bug rivers with the Danube being the largest and most important water and

sediment supplier, strongly influencing sedimentation in the north-western part of the basin (Panin and Jipa, 2002).

Sediment samples were collected during a one-time field campaign in July 2020 from two stations along the Romanian coast of the Black Sea (Figure 1), located 36.3 km apart, at water depths of 19.0 m (P8-20) (44°47,920'N; 29°37,850'E) and 27.0 m (CT-02) (44°17,420'N; 28°72,310'E), respectively (Figure 1). Station P8-20 is located within the area influenced by Danube freshwater (Figure 1).

Sediments were collected using a Van Veen grab sampler with a surface area of 0.135 m² (Todorova and Konsulova, 2005). The sedimentary material containing *M. arenaria* specimens from both sites consisted of mud, which derived from alluvial deposits composed of silt and clay particles. To evaluate the microbial communities, two sediment subsamples and two specimens of *M. arenaria* were collected from each station. The low number of samples was due to limitations related to sampling constraints. Typically, only one or two samples fall within habitats characteristic of this species, with no more than a few specimens captured per sample. After removing the sediment fraction that had been in contact with the sampling equipment to minimize contamination, the remaining sediment was transferred into sterile containers and stored at −20°C until further processing.

For bivalve DNA extraction, each selected specimen was washed with sterile water and preserved in 200 µl of Tris-EDTA buffer (pH 8.0) at −20°C (Ross et al., 1990).

2.2 Physicochemical parameters

In situ measurements of temperature, salinity, dissolved oxygen (DO) concentration, and pH in the bottom water level above the seabed were conducted using an EXO2 multi-parameter probe (YSI Incorporated, Yellow Springs, USA).

2.3 DNA extraction, 16S rRNA gene sequencing and sequence analysis

Genomic DNA was extracted from two *M. arenaria* individuals and two sediment samples from each sampling site. *Mya* specimens were rinsed with sterile water, dissected under aseptic conditions. DNA was isolated from the siphon, gills, and stomach of each individual using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following an optimized protocol that included an initial cell disruption step (Iancu et al., 2015). Tissue samples were resuspended in Tris-EDTA buffer (pH 8) and homogenized at 20°C for 12 min in a SpeedMill PLUS homogenizer at 50 Hz (Analytik Jena, Jena, Germany), with 5 ZR BashingBead 0.2 mm lysis matrix (Zymo Research, Irvine, CA, USA), and subsequently processed according to the manufacturer's protocol. For sediment samples, DNA was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The V3–V4 hypervariable regions of the 16S rRNA gene were amplified using the primer pair 341F/805R

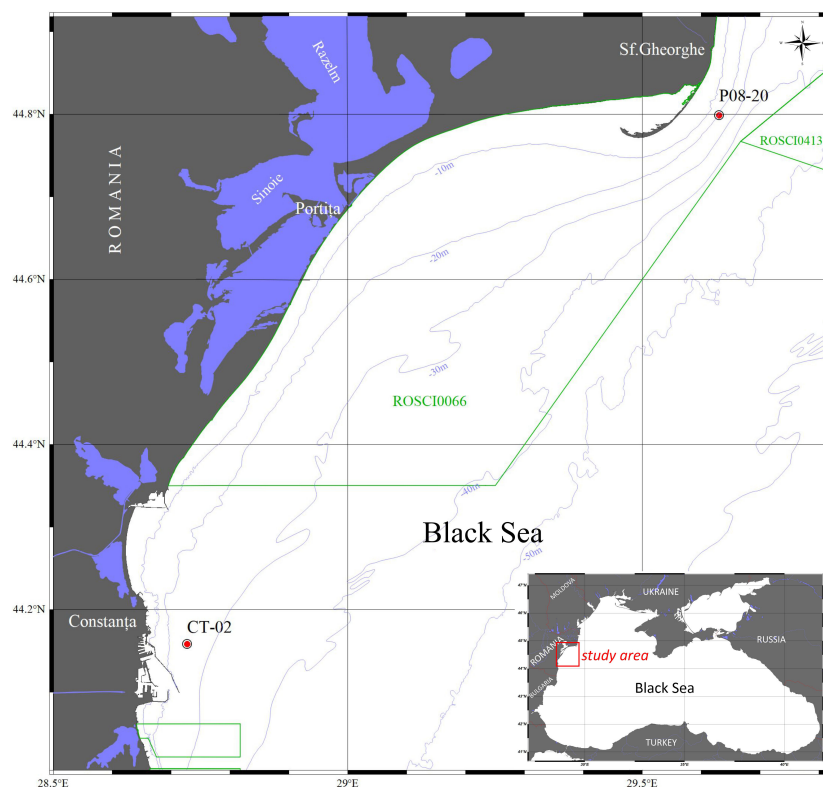


FIGURE 1

Map of the Romanian sector of the Black Sea showing the study area and sampling stations CT-02 and P08-20.

(Takahashi et al., 2014) and sequenced on an Illumina MiSeq platform with 300 bp paired-end reads (Macrogen, Seoul, South Korea).

The resulting DNA sequences were processed using the DADA2 package (v1.8) implemented in R (v4.0.2) (Callahan et al., 2016). After removing the forward and reverse primer sequences with cutadapt (v4.2.2) (Martin, 2011), the reads were trimmed and filtered (Lu et al., 2023). Amplicon sequence variants (ASVs) were inferred from the de-replicated sequences, and chimeras were removed using the consensus assignment method (Lu et al., 2023). Taxonomic identification of ASVs was performed using the SILVA v138 16S rRNA database (silva.nr. v138). Analyses were conducted using MicrobiomeAnalyst 2.0 (Lu et al., 2023). Standard data filtering procedures were applied to improve the quality of downstream analyses. A low-count filter was implemented using a minimum threshold of four reads, retaining only those features present in at least 20% of the samples (prevalence filter). Additionally, a low-variance filter was applied to remove features showing minimal variation across experimental conditions, based on the interquartile range (IQR). These filters aimed to eliminate low-abundance and noninformative features, which are often the result of sequencing noise or background contamination.

The 16S rRNA gene sequences of bacteria colonizing *M. arenaria* and associated sediments were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA922888.

Pattern search analysis based on Spearman correlation (Legendre and Legendre, 2012) was performed to assess whether specific taxa from particular organs and surrounding sediments were consistently associated, thereby enabling the identification of statistically significant relationships between data matrices.

The estimation of potential microbial functions was conducted by assessing gene abundances according to KEGG metabolism, using the TAx4Fun2 program (Lu et al., 2023).

2.4 Statistical analyses

Alpha and beta diversity was calculated using the pyloseq package (McMurdie and Holmes, 2013). Alpha diversity of ASVs was assessed using the Chao1, Shannon, Evenness, and Fisher indices. Bray–Curtis dissimilarity was used to assess beta diversity by comparing diversity between samples and microbial communities. The ordination-based method, Principal Coordinate Analysis (PCoA), was used to visualize the matrix in a 2-D plot, where each point represents the entire microbiome of a single sample. The statistical significance ($p < 0.05$) of the clustering patterns in ordination plots was evaluated using permutational ANOVA (PERMANOVA) and analysis of group similarities (ANOSIM). Statistical analyses were performed using MicrobiomeAnalyst 2.0 (Lu et al., 2023). To compare alpha diversity indices (Chao1, Shannon, and Fisher) among groups

(organs and sediments), the non-parametric Kruskal–Wallis test was applied, as it does not require assumptions of normality or homogeneity of variances. When significant differences were found ($p < 0.05$), *post hoc* multiple comparisons were performed using Dunn's test with Bonferroni correction to control for Type I error. All analyses were conducted in R (v4.5.1) using the FSA and PMCMRplus packages (Mangiafico, 2015; Pohlert, 2014).

LDA Effect Size (LEfSe) was based on the non-parametric Kruskal–Wallis test, which identifies significant differences in terms of taxa abundance across the analyzed sample types and ranks taxa according to the Linear Discriminant Analysis (LDA) (Segata et al., 2011). The Student's *t*-test was employed to statistically compare the mean gene abundance across samples (Mishra et al., 2019).

3 Results

3.1 Environmental characteristics of *Mya arenaria* habitat

In situ measurements of water parameters—temperature, salinity, dissolved oxygen (DO), and pH—at the two sampling stations (P8–20 and CT-02) revealed minimal variation in temperature and salinity, ranging from 11.01°C to 11.96°C and 17.92 PSU to 18.26 PSU, respectively (Table 1). A slightly alkaline pH was recorded at both sites, with an average of 8.09 ± 0.15 across the two stations (Table 1). Meanwhile, a lower DO value (4.2 mg L^{-1}) was recorded at the shallower P8–20 station, located under the Danube Delta influence area as compared to CT-02 (6.1 mg L^{-1}) (Table 1). These data suggest an overall similar physicochemical environment of the bivalve habitat across both sampling sites, regardless of water depth.

3.2 Bacterial diversity colonizing the organs of *M. arenaria* and sediments

To assess the microbial diversity and composition of *M. arenaria* organs and the surrounding environment, 14 DNA samples obtained from the siphon (4), gills (4), stomach (2), and sediments (4) were analyzed, based on sequencing quality of their corresponding 16S rRNA gene amplicons (Table 2).

Illumina sequencing of the 16S rRNA gene revealed a total of 1,073,832 DNA sequences and 684,356 normalized sequences, corresponding to 2,630 unique bacterial amplicon sequence variants (ASVs), ranging from 401 (sample MA16) to 41 (sample MA12), with an average of 187.9 ASVs.

Rarefaction curves for all analyzed samples from the P8–20 and CT-02 stations indicated a fully characterized bacterial community, capturing a representative segment of the microbial diversity within bivalve organs and sediments (Supplementary Figure S1).

Alpha diversity analysis based on the Shannon index revealed significant differences between the tissue samples and sediments ($p=0.0105$; [ANOVA] df -value=3) (Figure 2A, Table 2). Sediment-associated bacterial communities from both locations exhibited high microbial diversity, with Shannon values ranging from 5.55 to 5.76, and a mean of 5.68 ± 0.09 (Table 2). In host tissues, the Shannon diversity index for bacterial communities ranged from 2.99 to 4.62 in the siphon (mean: 3.75 ± 0.67) and from 2.57 to 4.53 in the gills (mean: 3.41 ± 0.81), while the stomach-associated bacterial communities ranged from 3.73 to 4.53, with a mean of 3.94 ± 0.3 (Figure 2A, Table 2). Although the highest mean was observed in the stomach, followed by the siphon and then the gills, the differences were not statistically significant (Figure 2A, Table 2). The corresponding Chao1, Shannon, and Fisher diversity metrics reflected a similar trend (Chao1 $p=0.0136$; [ANOVA] df -value=3; Shannon $p=0.0105$; [ANOVA] df -value=3; Fisher $p=0.0078$; [ANOVA] df -value=3), indicating lower diversity in tissue-colonizing bacteria compared to surrounding sediments (Table 2; Supplementary Tables S1, S2). In contrast, no statistically significant differences were observed between these communities across depths ([*t*-test] statistic=−0.375, $p=0.714$) (Figure 2B).

The multidimensional beta diversity analysis of bacterial communities, based on sample type, revealed significant differences in microbial composition. The PERMANOVA and ANOSIM results supported this finding: PERMANOVA indicated that approximately 47% of the variation ($R^2=0.468$) was explained by differences between tissues (gills, siphon, stomach) and sediments, underscoring the key role of habitat in structuring these communities. Additionally, ANOSIM ($R=0.564$) confirmed clear segregation between groups, with greater homogeneity within each sample type (e.g., sediment samples) than between sample types. The accompanying PCoA plot, in which axes 1 and 2 collectively explain approximately 62% of total variability, shows distinct clustering between tissue and sediment samples. The sediment samples appeared more homogeneous, indicating lower internal variability compared to mollusk tissues (Figure 3).

3.3 Taxonomic profile of bacteria associated with *M. arenaria* (siphon, gills, stomach) and sediments

The bacterial taxonomic assignment of *M. arenaria* organs revealed a diverse community encompassing 16 phyla, 23 classes,

TABLE 1 Physicochemical parameters of seawater from the sampling sites.

Station	Temperature (°C)	Salinity (PSU)	DO (mg L^{-1})	pH	Depth (m)
P8-20	11.96	17.92	4.2	7.99	19
CT-02	11.01	18.26	6.1	8.2	27

TABLE 2 Number of ASVs and alpha diversity indices of bacterial communities from *M. arenaria* organs and associated sediments, with statistical comparisons (Kruskal–Wallis test, *p*-values).

Station	Sample	Sample type	Total sequence	Normalized sequences	ASV (filtered)	Alpha Diversity index		
						Chao1	Shannon	Fisher
P8-20	MA1	siphon	50,197	24,921	133	133	3.78	19.51
	MA4		40,791	26,776	167	167	4.62	24.54
CT-02	MA9		74,280	58,169	57	57	2.99	6.40
	MA12		5,321	2,811	41	41	3.60	7.20
P8-20	MA2	gills	72,457	61,742	75	75	3.30	8.44
	MA5		94,449	88,153	60	60	2.57	6.29
CT-02	MA10		72,791	58,659	150	150	3.27	18.77
	MA13		88,868	54,475	216	216	4.53	29.13
P8-20	MA6	stomach	72,027	37,723	70	70	3.73	8.37
CT-02	MA11		96,100	50,067	181	181	4.16	24.26
P8-20	MA7	sediments	102,177	50,820	336	336	5.55	51.75
	MA8		106,297	57,978	380	380	5.71	58.99
CT-02	MA15		90,801	48,422	363	363	5.70	57.26
	MA16		107,276	63,640	401	401	5.76	61.30
siphon vs gills vs stomach vs sediments						<i>p</i> =0.0136	<i>p</i> =0.0105	<i>p</i> =0.0078
ANOVA Degrees of freedom (df)						df=3	df=3	df=3
Kruskal-Wallis chi-squared						H=10.68	H=11.23	H=11.89

34 orders, 41 families, and 43 genera in the siphon. The gill microbiome comprised 15 phyla, 21 classes, 33 orders, 39 families, and 35 genera, whereas the stomach-colonizing bacteria were assigned to 14 phyla, 22 classes, 30 orders, 29 families, and 31 genera. Meanwhile, the sediment microbiota consisted of 20 phyla, 38 classes, 50 orders, 48 families, and 54 genera (Supplementary Table 1).

At the phylum level, siphon-associated communities were dominated by *Verrucomicrobiota* (28.2%), *Spirochaetota* (26.5%), *Pseudomonadota I* (13%), and *Desulfobacterota* (8.7%) (Figure 4A). The gill-associated bacteria were dominated by *Verrucomicrobiota* (48.4%), followed by representatives of *Spirochaetota* (26%), *Pseudomonadota* (7.9%), and *Campylobacterota* (7.1%). In contrast, the stomach was dominated by *Spirochaetota* (32%), along with *Verrucomicrobiota* (23.5%), *Planctomycetota* (12.9%), and *Pseudomonadota* (9.6%) (Figure 4A). Meanwhile, sediment communities showed comparable proportions of *Desulfobacterota* (27.9%) and *Proteobacteria* (27%), along with *Bacteroidota* (9.3%) and *Chloroflexi* (7.4%) (Figure 4A).

The composition of bacterial classes (Supplementary Figure S2) differed substantially among tissues and environmental samples. While *Verrucomicrobiae* (28.2%) and *Spirochaetia* (26.5%) were the dominant taxa in the siphon, and the gills were primarily composed of *Verrucomicrobiae* (48.4%) and *Spirochaetia* (26.1%), the stomach hosted a bacterial community dominated by *Spirochaetia* (31.9%) and *Verrucomicrobiae* (23.4%) along with a notable contributions

from *Planctomycetes* (12.8%) and *Campylobacteria* (9.5%). In contrast, the sediment microbiome was predominantly composed of *Gammaproteobacteria* (25.4%) and *Desulfobulbia* (17.5%), with notable contributions from *Bacteroidia* (9.2%) and *Anaerolineae* (6.9%) (Supplementary Figure S2).

At the order level (Figure 4B), bacteria colonizing the siphon were dominated by *Spirochaetales* (26.2%), followed by *Verrucomicrobiales* (24.7%), *Desulfobulbales* (6.2%), *Clostridiales* (5.6%), *Enterobacterales* (4.7%), and *Planctomycetales* (3.3%). The gills and stomach samples were mainly composed of *Verrucomicrobiales*, accounting for an average of 45.9%, and 17.8%, respectively. *Campylobacteriales* were also highly represented in these two organs, with average relative abundances of 7.2%, and 9.5%, respectively. Moreover, notable abundances of *Spirochaetales* (27.4%) were observed in the gills, with lower contributions from *Enterobacterales* (2.4%) and *Bacteroidales* (1.9%). Meanwhile, *Planctomycetales* (8.8%), *Chthoniobacterales* (5.2%), *Enterobacterales* (4.7%), and *Synechococcales* (3.8%) were better represented in the stomach (Figure 4B). For the sediment samples, *Desulfobulbales* dominated (16.9%), followed by representatives of *Steroidobacterales* (9.6%), *Flavobacteriales* (8.0%), and *Pseudomonadales* (5.2%) (Figure 4B).

The taxa belonging to the families *Spirochaetaceae* and *Rubritaleaceae* families were identified at high abundances in the three analyzed organs (Supplementary Figure S3). Thus, in the siphon samples, they accounted for an average of 26.2% and 24.7%,

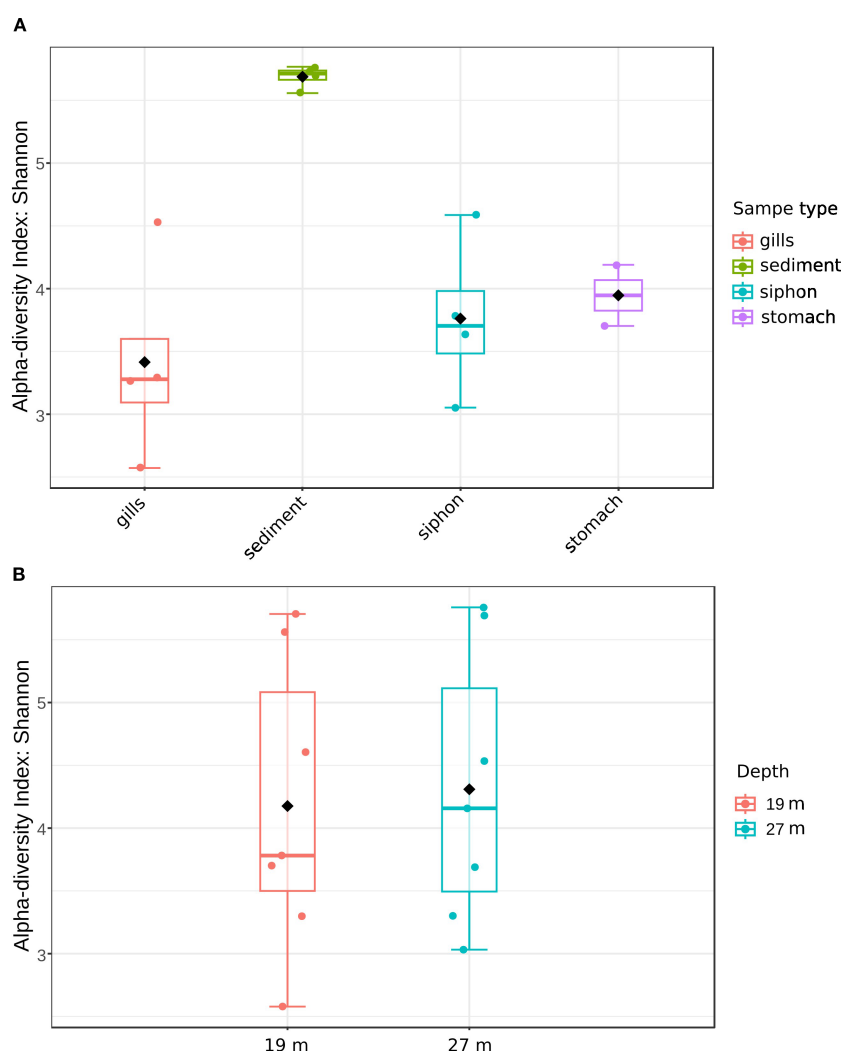


FIGURE 2

Alpha diversity of bacterial communities from *M. arenaria* organs and sediments based on Shannon index. (A) sample type (siphon, gills, stomach, sediments); (B) depth (m) of the sampling site.

respectively; in the gills, 45.9% and 27.4%, and in the stomach, 32.5% and 17.7%. *Desulfobulbaceae* (4.2%) and *Enterobacteriaceae* (4.3%) were better represented in the siphon, *Arcobacteraceae* (4.2%) in gills, while *Sulfurimonadaceae* (7.8%) and *Chthoniobacteraceae* (5.2%) exhibited higher relative abundances in the stomach. Meanwhile, bacterial species of *Desulfobulbaceae* (12.1%) dominated the sediment samples, followed by *Woseiaceae* (9.6% average) and *Flavobacteriaceae* (8.0%) (Supplementary Figure S3).

Clear differences were observed in the abundance of bacterial genera across the bivalve organs and associated sediments (Figure 4C). In the siphon, the community was dominated by unassigned sequences (44.1%), followed by the genus *Persicirhabdus* (24.9%), with minor contributions from *Clostridium_sensu_stricto_1* (5.7%) and *Escherichia/Shigella* (4.5%). The gills showed a high abundance of *Persicirhabdus* (45.7%) and unassigned sequences (39.0%), while *Sulfurimonas* (2.2%) and *Poseidonibacter* (2.1%) emerged as the next most

relevant genera. In the stomach, unassigned sequences were predominant (50.6%), followed by *Persicirhabdus* (17.7%) and *Sulfurimonas* (7.8%), along with LD29 (5.2%), a poorly characterized genus. Finally, in sediments, unassigned sequences dominated (52.9%), followed by *Woseia* (9.4%), *Ilumatobacter* (4.8%), and *Halioglobus* (3.5%), genera associated with marine environments and sulfur cycling (Figure 4C).

Heatmap analysis of the distribution of bacterial phyla in clam organs and sediments highlighted similarities and differences among bacterial communities according to the sample type and water depth (Figure 5). The analysis revealed that bacteria colonizing the organs of *M. arenaria* formed a distinct cluster compared to those associated with sediments (Figure 5). Moreover, no association pattern among these communities with respect to water depth was observed (Figure 5).

Pattern search analysis of bacteria phyla from clam organs and sediments (Figure 6) revealed a positive correlation between *Verrucomicrobiota* and gills, as well as between sediment samples

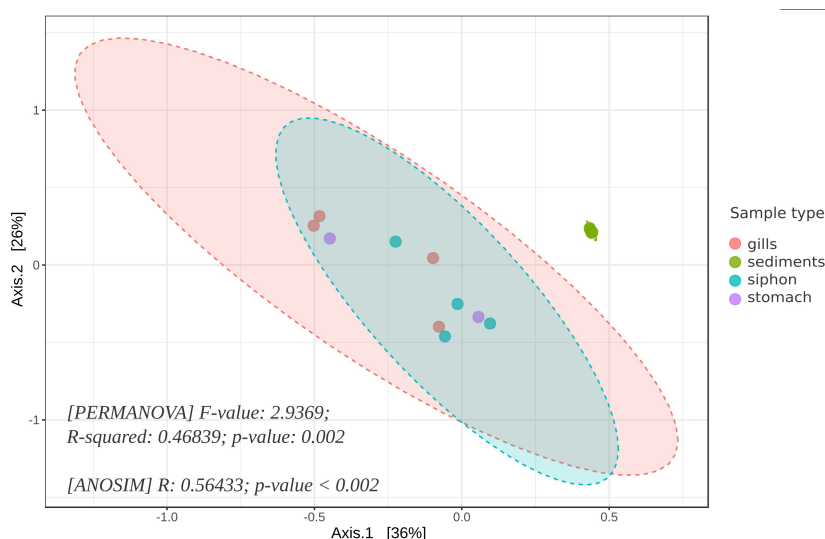


FIGURE 3

PCoA analysis of the beta diversity of bacterial communities from *M. arenaria* organs (siphon, gills, stomach) and adjacent sediments, visualized at ASV level; ellipses represent 95% confidence intervals for each sample group, and statistical significance was confirmed by PERMANOVA ($F=2.94$, $R^2=0.47$, $p=0.002$) and ANOSIM ($R=0.56$, $p<0.002$).

and *Latescibacterota*, *Sva0485*, *Chloroflexi*, *Myxococcota*, *Nitrospirota*, *Patescibacteria*, and *Desulfobacterota*, which were either absent or present at very low abundances in the analyzed organs. *Campylobacterota* was also positively correlated with the stomach samples (Figure 6). Conversely, a negative correlation was observed between phyla *Planctomycetota* and *Firmicutes* and the gill samples, as well as between *Cyanobacteria* and the siphon samples (Figure 6).

The LDA Effect Size (LefSe) analysis of microbial taxa with significant differential abundance among the sample types (Supplementary Figure S4) identified *Spirochaetota*, *Planctomycetota*, and *Cyanobacteria* as significant biomarkers differentiating the analyzed sample types (LDA score >3), highlighting their dominance in the stomach. Moreover, the prevalence of other phyla including *Desulfobacterota*, *Pseudomonadota*, *Bacteroidota*, *Chloroflexi*, and *Actinobacteriota* (LDA score >3), was observed in the sediment samples (Supplementary Figure S4).

3.4 Predicted functional role of the microbiome of *M. arenaria* from different organs and associated sediments

The 16S rRNA gene sequencing data was used to estimate the functional profile of the corresponding bacteria based on sequence similarities to organisms with fully sequenced and annotated genomes, using Tax4Fun with SILVA annotated features for functional profiling.

The predicted functional profile analysis showed a comparable distribution of the relative abundance of various genes based on Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways (Figure 7). In this respect, genes related to carbohydrate, amino acid, and energy metabolism were prevalent across all

samples, followed by those involved in the metabolism of cofactors, vitamins, and nucleotides, which showed comparable values between organs and sediments (Figure 7). Although not among the dominant pathways, several associated with xenobiotic biodegradation and antibiotic biosynthesis were identified (Figure 7). Thus, the analysis revealed a slight increase in the expression of genes associated with most of the identified degradation pathways within organs (e.g., degradation of toluene, ethylbenzene, xylene, chlorocyclohexane, and chlorobenzene), suggesting differential metabolic activity between the two types of matrices analyzed (Figure 7). With regard to antibiotic synthesis, no major differences in gene abundances were observed between organs and sediments, with the dominant functions being related to streptomycin and monobactam biosynthesis, which were slightly more prevalent in sediment-colonizing bacteria. Meanwhile, the analyzed organs showed high abundances of genes involved in penicillin and cephalosporin biosynthesis (Figure 7).

Although the beta diversity analysis (Figure 3) revealed significant differences between clam tissues and sediment microbiota, their predicted functions based on 16S rRNA gene sequences suggested only limited variation between the two matrices. In this regard, further investigations using metagenomic and metatranscriptomic analyses are required to gain deeper insight into the functional variability of these communities (Aguilar-Pulido et al., 2016), which may provide a more detailed understanding of their metabolic profiles.

4 Discussion

This study aimed to characterize the bacterial communities of the bivalve *M. arenaria* collected from the Romanian part of the

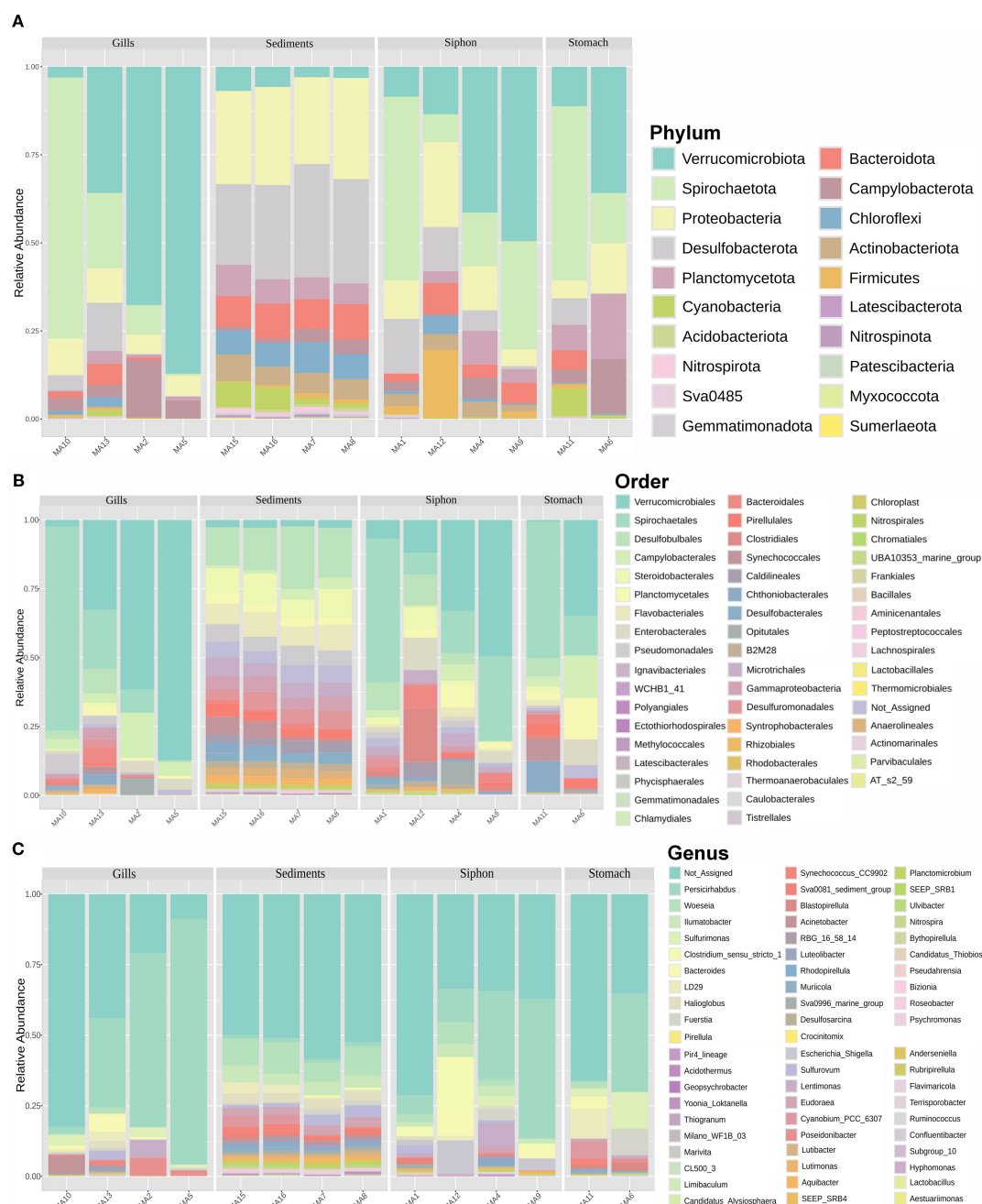


FIGURE 4

Relative abundance profile of bacterial taxa associated with *M. arenaria* organs (siphon, gills, stomach) and surrounding sediments. (A) phyla; (B) order; (C) genera.

Black Sea, and is the first to examine the microbiome of different organs (siphon, gills, and stomach) of this marine invertebrate, as well as the associated sediments.

Previous research on *M. arenaria*-associated bacteria has primarily focused on microbial contamination, including studies on the kinetics of coliform bacteria uptake and removal by this bivalve (Cabelli and Heffernan, 1970), and, more recently, reports of significant contamination with pathogens such as *Arcobacter*, *Cryptosporidium parvum*, *Giardia*, and *Salmonella* in clams collected from the north shore of the St. Lawrence River (Quebec,

Canada) (Lévesque et al., 2006). The present study applied culture-independent Illumina sequencing of the 16S rRNA gene to characterize the taxonomic and functional diversity of bacterial communities colonizing the siphon, gills, and stomach of *M. arenaria*, as well as associated sediments from two locations in the Black Sea. This represents a more comprehensive and targeted molecular characterization compared to a previous NGS study of *M. arenaria* microbiome, which only characterized the bacterial diversity of the whole organisms collected from two locations on the east coast of Canada (Liu et al., 2020). Moreover, this study

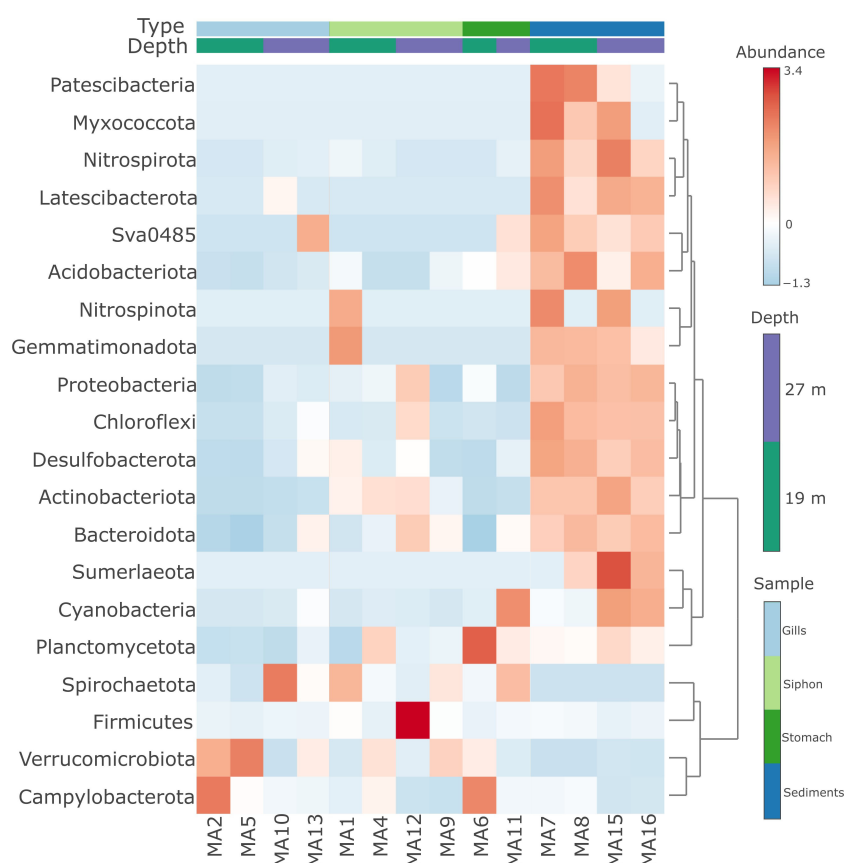


FIGURE 5

Heatmap distribution of bacterial phyla from *M. arenaria* organs (siphon, gills, stomach) and sediments, in relation to water depth.

investigated the hypothesis that *M. arenaria* hosts a microbiome that may help it cope with stressful estuarine conditions and organic pollution, including bacterial contaminants. This molecular approach also enabled the identification of bacterial genes potentially involved in diverse metabolic functions, including antibiotic resistance, providing insight into functional traits that allow the clam to cope with environmental stressors.

Our data showed that the bivalve microbiota was well differentiated from that of its physical habitat, and exhibited compositional variations depending on the organ, with greater taxonomic diversity (19 phyla) in the sediments as compared to the bivalve organs (14–15 phyla), independent of water depth. The phylum *Verrucomicrobiota* dominated the siphon and gill samples, *Spirochaetota* was the most abundant in the stomach, and the sediment communities were mainly composed of *Desulfobacterota*.

No significant differences in bacterial diversity were observed between samples from different depths, likely reflecting similar environmental conditions across sites. All samples were collected from circalittoral sandy mud habitats, which are characterized by benthic communities dominated by detritivores and suspension feeders such as oligochaetes, polychaetes, mollusks, and bacterivorous nematodes (Nenciu et al., 2023; Mureșan et al., 2019; Teacă et al., 2019, 2020). These results align with other studies showing that microbial communities in similar habitats

are comparable, even across different geographical regions (Laubert et al., 2009).

Recent investigations in the Black Sea revealed the dominance of *Actinobacteriota* and *Chloroflexi* in sediments from areas with physico-chemical water characteristics and substrate composition comparable to those observed in our research (Menabit et al., 2024). While these bacteria were also detected, the most abundant taxa colonizing the sediments in this study were assigned to *Pseudomonadota* and *Desulfobacterota*. Shifts in microbial composition might be attributed to the distinct habitat types (Teacă et al., 2020), as such changes in the structure of the zoobenthic community could influence microbial populations. While prior research (Reveillaud et al., 2014) has demonstrated that microbial communities often reflect host and environmental differences across large spatial scales, our study found relatively minor differences in bacterial composition between the two sampling sites, suggesting limited spatial variation in this context.

Bivalves, like many other organisms, host microbial communities that are specific to various organs and tissues. Previous data have reported the digestive system as containing the highest concentrations of bacteria, particularly the stomach, gastric juice, crystalline style and digestive diverticula (Kueh and Chan, 1985). The results obtained in this survey indicated a specific microbiota in this bivalve, distinct from its physical habitat, with compositional variations depending on the organ. Thus,

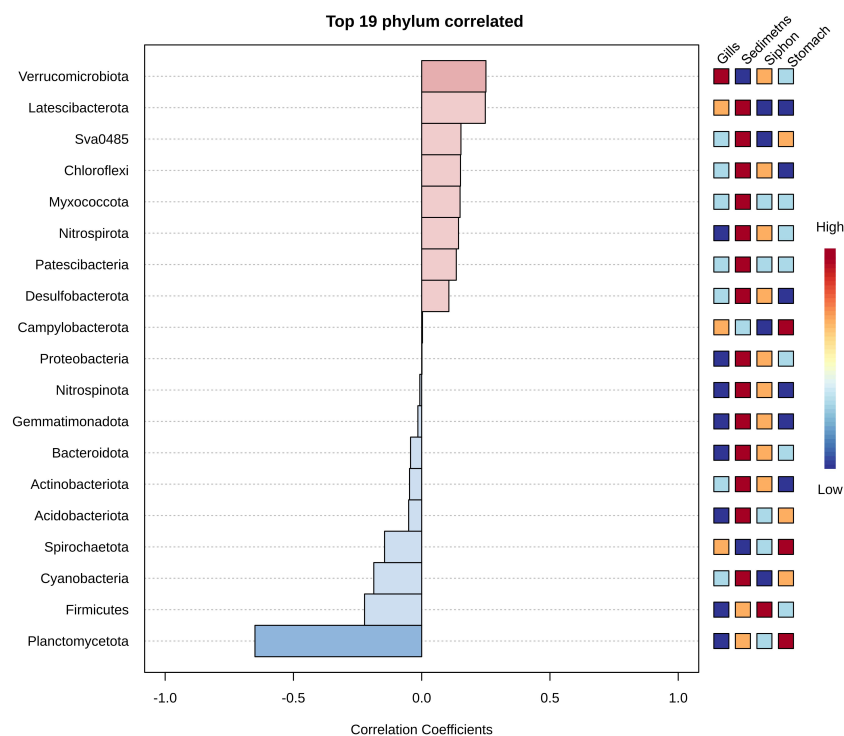


FIGURE 6

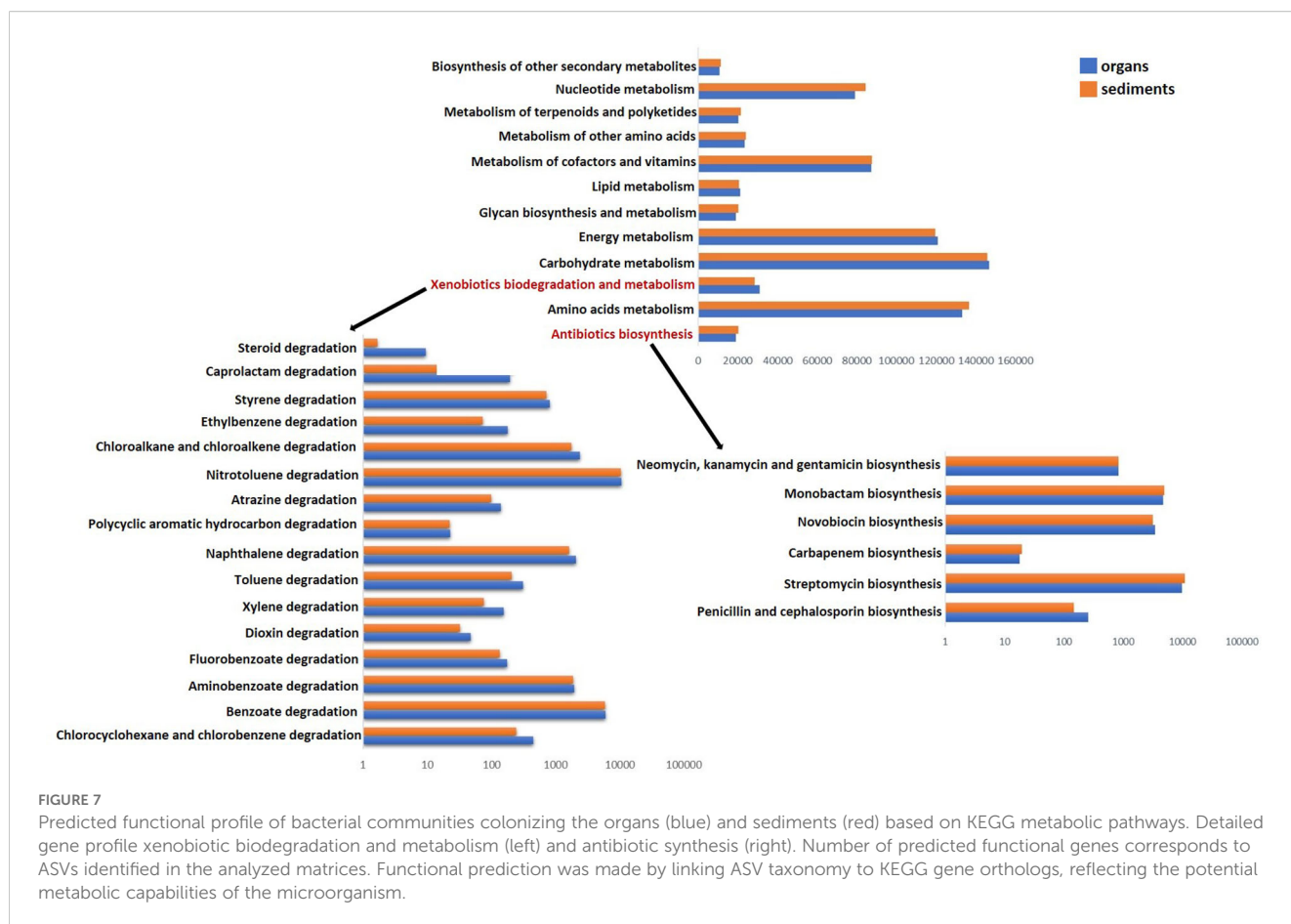
Phylum-level correlation analysis between bacterial taxa and sample types in *M. arenaria*, showing the strength and direction of associations for the top 19 most correlated phyla; positive correlations (red) indicate enrichment in specific organs or sediments; negative correlations (blue) indicate depletion, as determined by Spearman's rank correlation.

representatives of the order *Spirochaetales* showed the highest abundances in siphon and gill samples, with the *Verrucomicrobiales* group being better represented in gills. Taxa belonging to *Spirochaetales* have also been identified in the digestive tract of invertebrates such as arthropods (Berlanga et al., 2007) and mollusks (Romero and Espejo, 2001), as well as in bivalve gills (Duperron et al., 2007). A recent study in New Zealand showed these bacteria to be abundant in the siphon and digestive glands of the bivalve *Paphies australis* (Biessy et al., 2020), consistent with our data, which revealed a high representation of *Spirochaetales* taxa in the siphon samples. Bacteria classified in the order *Verrucomicrobiales* have been identified in various environments such as the water column (Yoon et al., 2007), sediments (Yoon et al., 2008), in association with marine invertebrates, such as the polychaete *Periserrula leucophryna* (Yang et al., 2007) and different organs (gills, stomach, foot) of the bivalve *M. galloprovincialis* (Musella et al., 2020). The results also indicated a statistical correlation between the phylum *Planctomycetales* and stomach samples, consistent with data on the stomach microbiome of *C. gigas*, which showed a high abundance of taxa belonging to this group (Offret et al., 2020).

The present data revealed distinct, organ-specific distributions of bacterial taxa within *M. arenaria*. Members of the order *Bacteroidales* were predominantly associated with the siphon, while bacteria from *Campylobacteriales* and *Synechococcales* exhibited higher relative abundances in the stomach samples. The enrichment of *Bacteroidales* is consistent with the high

representation of this phylum in other marine invertebrates, including the crab *Callinectes sapidus* (Givens et al., 2013), the gastropod *Haliotis diversicolor* (Zhao et al., 2018), and the bivalve *M. galloprovincialis* (Musella et al., 2020). Similarly, *Campylobacteriales* that prevailed in the stomach of *M. arenaria* have been reported in the digestive tracts of various marine organisms, such as the sea urchin *Lytechinus variegatus* (Hakim et al., 2015) and the ascidian *Ciona intestinalis* (Dishaw et al., 2014), suggesting a possible functional role in digestion. The presence of *Synechococcales* in stomach tissues may be linked to the dietary habits of *M. arenaria*, as these cyanobacteria are known to constitute a significant nutritional component for other marine taxa, including sipunculid worms (Li et al., 2023a; Liu et al., 2023) and bivalves (Qiao et al., 2022).

Despite variations in composition, our research showed that the *Verrucomicrobiota* and *Spirochaetota* phyla exhibited high abundances in all analyzed organs. In addition, a notable proportion of sequences belonging to *Planctomycetota* was recovered from the stomach samples. A study conducted at two locations on the East Coast of Canada demonstrated that soft-shell clams from Nova Scotia had a higher abundances of *Pseudomonadota* and *Acidobacteria*, but a lower abundance of *Actinobacteria* than those from Quebec. Furthermore, samples from Nova Scotia exhibited greater microbial diversity than those from Quebec (Liu et al., 2020). These findings suggest that environmental factors may shape the microbial communities of this bivalve, influencing both composition and diversity.



The genus *Persicirhabdus* encompasses nitrate-reducing bacteria (Yoon et al., 2008), which, under certain conditions, such as low oxygen levels, can reduce nitrates to nitric oxide (Strahl and Abele, 2020). Studies have shown that the clam *Arctica islandica* may harbor nitrate-reducing bacteria, and that nitric oxide could play a crucial role in reducing the metabolic activity of *A. islandica* during self-burial and shell closure. Under these conditions, nitric oxide contributes to the initiation of metabolic depression, which may help conserve energy during hypoxia or anoxia and protect against oxidative stress (Strahl and Abele, 2020). Accordingly, the high abundance of these bacteria in the analyzed organs of *M. arenaria* could support the survival and adaptation of this bivalve in the low-oxygen conditions of the investigated area.

The genus *Sulfurimonas* is characterized as sulfur-oxidizing bacteria, capable of growing chemoautotrophically with reduced sulfur compounds, such as sulfide, thiosulfate and elemental sulfur (Sievert et al., 2008), and has been isolated from various environments, including coastal marine sediments (Hoor, 1975; Cai et al., 2014; Wang et al., 2020), polychaete nests (Inagaki et al., 2003; Takai et al., 2006), and deep-sea hydrothermal vents (Hu et al., 2021). While sulfur-oxidizing bacteria are typically found in the gills of marine bivalves (Dando et al., 1985; Caro et al., 2007), recent investigations have revealed a persistent population of these microbes in the digestive tract of adult hosts lucinid clam *Loripes orbiculatus*, which contribute to the host's nutrition by fixing

carbon from sulfide oxidation (Alcaraz et al., 2024). These findings are consistent with our investigations, where we detected higher abundances of *Sulfurimonas* colonizing the stomach samples, further suggesting a role of these bacteria in digestion or other processes that contribute to the host's nutrition.

Bacteroides, identified in all analyzed organs of *M. arenaria*, was previously documented in the digestive tract of the mussel *M. edulis* and the oyster *C. virginica*, and plays an important role in digestion, including the breakdown of complex carbohydrates and the production of essential amino acids (Pierce and Ward, 2019).

Poseidonibacter is considered a synonym or subgroup within the genus *Arcobacter* (On et al., 2021), members of which are known pathogens linked to human and animal diseases, previously detected in various water bodies, including wastewater, seawater, and freshwater systems, often associated with fecal contamination (Ghaju Shrestha et al., 2022), as well as colonizing soft-shell clams collected from the north shore of the St. Lawrence River, and other bivalves such as *M. galloprovincialis* (Ottaviani et al., 2016). In our study, we observed high abundances of these bacteria in the gills of *M. arenaria* specimens collected from the station located in the Danube influence area, which was not surprising given that the river is permanently subject to anthropogenic pressures such as industrial activities, intensive farming, and agricultural runoff (Gasparotti, 2014). Moreover, the *Escherichia/Shigella* genera were identified in all analyzed organs. *Escherichia* includes both

commensal and pathogenic species (Braz et al., 2020), whereas *Shigella* is highly infectious and responsible for significant global morbidity and mortality (Schroeder and Hilbi, 2008). Bivalves, as filter-feeding organisms, concentrate bacteria from the environment and serve as suitable bioindicators of water contamination. Consequently, the presence of these genera in bivalve tissues may pose serious public health risks through seafood consumption and highlight underlying threats to ecosystem health, including microbial pollution and reduced water quality.

Unlike the microorganisms associated with *M. arenaria*, those colonizing the sediments were mainly represented by taxa belonging to the orders *Desulfobulbales*, *Steroidobacterales*, and *Flavobacteriales* orders. The high abundance of the *Desulfobulbales* group, mainly composed of sulfur-reducing bacteria thriving in anoxic environments (Ward et al., 2021), could be correlated with the low dissolved oxygen concentrations characterizing the Romanian part of the Black Sea. These particularities could also explain the presence of taxa belonging to *Steroidobacterales* in sediments, which are known for their ability to oxidize sulfur (Mußmann et al., 2017). Previous studies have demonstrated that *Chloroflexi* species account for 25.5% to 41.3% of total 16S rRNA sequences in marine sediments globally (Hoshino et al., 2020). Some representatives of this phylum are also known for decomposing organic matter (Landry et al., 2017). In this study, the prevalence of this group in sediment samples could be associated with the marine habitat, characterized by high organic matter content, from which the samples were collected (Bucşe et al., 2020; Teacă et al., 2020). Moreover, the genus *Woeseia* was consistently detected across all sediment samples. This group comprises halotolerant bacteria capable of sulfur oxidation and denitrification (Du et al., 2016; Mußmann et al., 2017) and has previously been isolated from both coastal and deep-sea surface sediments (Mußmann et al., 2017; Hoffmann et al., 2020).

A preliminary assessment of the functional profile of the microbiome of the bivalve *M. arenaria* and adjacent sediments was performed by determining the functional diversity of the bacterial communities based on 16S rRNA gene sequencing data, using the Tax4Fun platform (Langille et al., 2013). While this tool cannot replace the assessment of gene functionality through metagenomic analysis, studies have shown that it can provide insight into the functions of microorganisms in diverse habitats such as biofilms (Koo et al., 2017), the water column (Bowman and Ducklow, 2015), and sediments (Su et al., 2018). However, the effectiveness of this method depends on the genomic information available in databases, which in some cases does not reflect the functional diversity of the microorganisms in the investigated ecosystem (Wemheuer et al., 2020).

The data obtained in this study on the functional diversity of the bacterial communities associated with the bivalve *M. arenaria* and surrounding sediments are consistent with previous investigations emphasizing the role of marine bacteria in carbohydrate metabolism (Gavrilidou et al., 2020). Thus, studies have shown that bacteria associated with some bivalves produce enzymes that break down complex carbohydrates, and contribute to the immune response of these organisms (Dubé et al., 2019). In addition, representatives of the orders *Verrucomicrobiales* and *Bacteroidales*

orders, mainly identified in *M. arenaria* organs, are involved in the hydrolysis of various polysaccharides (Martinez-Garcia et al., 2012). Likewise, Dunkai et al. (2023) demonstrated that bacterial strains isolated from the digestive system of the bivalve *Crenomytilus grayanus* can degrade various nutrient substrates (sugars, amino acids, and polysaccharides) as well as xenobiotic substances (petroleum hydrocarbons, bisphenol A, and atrazine). Therefore, the presence of genes involved in xenobiotic metabolism in the microbiota of *M. arenaria* could play an important role in maintaining the health and adaptability of this species in diverse environments, including polluted areas. Several studies have highlighted the presence and potential of microbial communities colonizing marine sediments to degrade aromatic hydrocarbons, including toluene, ethylbenzene, xylene, chlorocyclohexane, and chlorobenzene (Moore et al., 2020; Suarez-Moo et al., 2020; Nikolova et al., 2021). Similar degradation pathways which have also been identified in the current study, highlighting the potential of these bacteria as valuable resources for bioremediation and the pharmaceutical industry. Moreover, research undertaken by Šyvokienė and Butrimavičienė (2013) demonstrated that bacteria associated with the digestive systems of swollen river mussels (*Unio tumidus*) and zebra mussels (*Dreissena polymorpha*) from the Curonian Lagoon (Winters et al., 2011) are involved in the biodegradation of petroleum hydrocarbons. Mollusks, including bivalves, are emerging as a promising source of novel marine bioactive compounds, with certain species containing substances that could have diverse applications across industries (Sousa and Hinzmann, 2020). The current investigation demonstrates that bacteria associated with both the organs of *M. arenaria* and the surrounding sediments harbor genes involved in antibiotic synthesis. This finding aligns with other research, such as studies from the Red Sea, where the detection of biosynthetic gene clusters polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) in marine invertebrate-associated bacteria indicates their potential to produce bioactive compounds, including antibiotics (El Samak et al., 2018).

5 Conclusion

This study revealed significant differences in bacterial community diversity between the organs of the bivalve *M. arenaria* and the adjacent sediments, with greater diversity in the sediments. The bivalve harbors a distinct microbiota that varies by organ and differs clearly from that of its physical habitat. Notably, the phylum *Verrucomicrobiota* predominated in the siphon and gills, while *Spirochaetota* were more abundant in the stomach. In contrast, *Desulfobacterota* dominated the sediment-associated microbial communities. *Persicirhabdus* bacteria were consistently abundant across all examined organs and may contribute to the clam's metabolic depression and adaptation to low-oxygen conditions. Additionally, sulfur-oxidizing *Sulfurimonas* species in the stomach likely contribute to the host's nutrition. Sediments, in contrast, were primarily colonized by *Woeseia* species, which are key players in the degradation of organic matter. Functional diversity analyses

suggested that bacteria inhabiting both bivalve tissues and adjacent sediments participate in diverse metabolic processes, with a particular emphasis on carbohydrate, amino acid, and energy metabolism. Several pathways were associated with xenobiotic degradation and antibiotic biosynthesis, highlighting the biotechnological potential of these microbial communities for applications such as bioremediation and pharmaceutical development.

This survey, providing the first organ-specific characterization of the microbiome associated with *M. arenaria* revealed clear structural differences among the siphon, gills, and stomach, as well as a distinct separation from the surrounding sediment microbial communities. The observed compositional divergence highlighted the presence of a specialized and compartmentalized microbiome within the bivalve, likely shaped by organ-specific functions and external environmental exposures. Notably, the internal microbial communities do not mirror the environmental microbiota, suggesting a non-random, potentially symbiotic assemblage with functional relevance to host biology. These results offer a foundation for future investigations into the ecological and physiological roles of the bivalve microbiome, particularly in the context of environmental stress and host adaptation. Further studies employing metagenomic and metatranscriptomic approaches will be essential to elucidate the mechanistic interactions between *M. arenaria*, its microbiota, and the surrounding habitat. Nevertheless, the small number of specimens analyzed could potentially bias interpretations of the structural and functional diversity within the bivalve microbiome.

Data availability statement

All data supporting the findings of this study are provided within the manuscript. The 16S rRNA gene sequences from this study were deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA922888 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA922888/>).

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

SM: Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. PL: Writing – review & editing, Writing – original draft, Software, Methodology. TB: Writing – review & editing, Resources. AT: Writing – review & editing, Software, Resources. MM: Writing – review & editing, Resources. CP: Writing – review & editing, Writing – original draft, Validation, Supervision, Formal Analysis, Conceptualization.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1659674/full#supplementary-material>

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