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Research Article

First Record on Microbial Colonies in Freshwater Sponges in East Java, Indonesia, and Their Estimated Pollutant Degradation Genes

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ABSTRACT

Sponges are known to harbor diverse and abundant microbial colonies. Nevertheless, studies on the diversity and abundance of microbial colonies in freshwater sponges have not been as extensive as those of marine sponges. This study investigates the microbial colonies of two freshwater sponge species, *Eunapius carteri* and *Oncosclera asiatica*, from the Kaliporong River in Indonesia. High microbial diversity was observed, with *E. carteri* harboring over 1,400 unique microbial species (Operational Taxonomic Units or OTUs) and *O. asiatica* hosting over 400. Proteobacteria were the dominant bacterial group in both sponges, comprising over 90 % of *O. asiatica* and over 50 % in *E. carteri* colonies. Functional profiling revealed a high potential for xenobiotic degradation in both sponge species, particularly through pathways involving Cytochrome P450 and the degradation of benzoate, caprolactam, and aminobenzoate. *O. asiatica* shows more of these degradation pathways than *E. carteri*. For example, benzoate degradation, involving over 60 genes or enzymes, was more pervasive in *O. asiatica* (5.81 %) than in *E. carteri* (5.03 %). These findings highlight the significant role of freshwater sponges in supporting diverse microbial populations with potential for bioremediation, particularly in polluted environments like the Kaliporong River. Further research is needed to understand the specific functions of these microbial colonies in freshwater sponges and their impact on the ecosystem.

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INTRODUCTION

Sponges are mostly found in the marine ecosystem, inhabiting both shallow and deep-sea environments. Notably, tropical regions, particularly Indonesian waters, are considered biodiversity hotspots for sponge exploration, surpassing sub-tropical and polar regions in species richness (Putra et al. 2023). According to the World Porifera Database (WPD, 9,490 sponge species have been identified to date, with estimates suggesting the actual number may surpass 20,000 due to many species remaining unidentified, cryptic, or difficult to classify (de Voogd et al. 2024). Research on sponges has gained momentum over the past two decades, partly because sponges represent the earliest branch of the animal lineage (Animalia) and exhibit a simple level of biological organization (Pisani et al. 2015; Adamska 2016; Feuda et al. 2017). Therefore, sponges are suitable for studying animal evolution and the origin of multicellular life. Sponges also possess an important role in ecology, especially in marine ecosystem. In addition to support coral reef survival, they serve as a bioeroder for coral restoration and recycles chemical elements (Pawlik & McMurray 2020). They can also serve as an alternative source of biochemical or biopharmaceutical compound (Rajendran 2016; see detail in Varijakzhan et al. 2021) as they harbor symbiotic organisms such as microbes.

Microbes are known as a diet component of sponges but many extracellular microbes also inhabit sponge tissues, living as symbiotic bacteria. According to the symbiotic relationships, sponges are categorized in two groups: High Microbial Abundance (HMA) and Low Microbial Abundance (LMA) sponges (Gloeckner et al. 2014; Moitinho-Silva et al. 2017). The difference between these two groups is that symbionts assist in the nourishment. HMA sponges have smaller filtering structures but process large volumes of water, while LMA sponges possess larger filtering organs (Poppell et al. 2014) as they have less symbiotic microbes. LMA sponges invest more energy in developing feeding structures since their nutrition needs are less supported by microbial symbionts. Meanwhile, HMA sponges receive substantial nutritional support from their abundant microbial symbionts and, as a result, do not require large filter-feeding structures. Proteobacteria, Poribacteria and Actinobacteria are well-known symbiotic microbes commonly found in sponges. These microorganisms are known to dominate in marine sponges, whereas freshwater sponges remain comparatively understudied.

Freshwater sponges are classified as a minor group of sponges that inhabit freshwater ecosystem and possess unique characters. A key feature of freshwater sponges is their ability to survive fluctuating or extreme environmental conditions, such as drought, and to disperse over long distances. This resilience is due to their production of gemmules—specialized asexual reproductive structures (see detail in Manconi & Pronzato 2016). Ecologically, freshwater sponges link energetic pathway between pelagic and benthic community in the freshwater ecosystem by hosting zoo-chlorella and serving as prey for spongivorous insects (e.g., Hall et al. 2021; Ruengsawang et al. 2022). Studies on microbes, especially bacteria group in freshwater sponges not as extensive as those on marine sponges. Similarly, knowledge of microbial symbionts in freshwater sponges remains limited to a few bacteria groups (Keller-Costa et al. 2014). The microbial symbionts in freshwater sponges are not as diverse as marine sponges (see review in Lo Giudice & Rizzo 2024; Setiawan et al. 2024). However, after utilization of next generation sequencing (NGS) on symbiotic bacteria diversity, freshwater sponges also harbor a comparable number of microbes with marine sponges; for example, in India, it is discovered that Firmicutes, Proteobacteria, Cyanobacteria and Actinobacteria were the most abundant groups of freshwater sponges (Gaikwad et al. 2016). The presence of Actinobacteria suggests that freshwater sponges also hold potential for the discovery of novel bioactive compounds.

There has not any study yet on freshwater sponges in Indonesia in terms of microbial diversity. For this reason, we aim to compare and explore community symbiotic bacteria from two well-identified freshwater sponges in East Java Indonesia namely as *Eunapius carteri* (Bowerbank, 1863) and *Oncosclera asiatica* (Manconi & Ruengsawang, 2012) that are categorized under order Spongillida (Manconi & Pronzato, 2002) based on profiling of 16S rRNA gene utilizing Illumina Novaseq platform. *E. carteri* is a, globular sponge that attaches to hard substrates in muddy environments, which has a flat, encrusting form and adheres to rocky substrates in river systems such as Kaliporong, near Surabaya city in East Java Province Indonesia (Setiawan et al. 2023).

MATERIALS AND METHODS

Materials

For microbiome isolation, DNA extraction buffer was made using Tris base (Himedia, Mumbai, India), EDTA (Biorad, California, US), sodium phosphate (Merck, Darmstadt, Germany), sodium chloride (Himedia, Mumbai, India), CTAB (Himedia, Mumbai, India), and proteinase K (Intron, South Korea). Sodium dodecyl sulphate (SDS), chloroform, isoamyl alcohol, isopropanol, ethanol, and distilled water were used for DNA extraction. MO-BIO PowerClean® DNA Clean-Up Kits (Qiagen, Germany) was used to purify and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, US) to quantify DNA extracts. Agarose gel and sodium borate (SB) buffer were used to assess DNA quality.

As for PCR, Phusion® High-Fidelity PCR Master Mix (Biolabs, New England) were used to amplify the DNA using 515F (5'-G T G C C A G C M G C C G C G G T A A) and 806R (5'-GGACTACHVHHHTWTCTAAT) primer. Agarose, Tris Acetate EDTA (TAE), florosafe DNA stain (1st BASE), 1 Kb Plus DNA ladder (Invitrogen, Thermo Fisher Scientific, US), and DNA loading dye (Thermo Fisher Scientific, US) were used for electrophoresis. Moreover, Mo-Bio UltraClean PCR Clean-Up Kit (Qiagen, Germany) was used for amplicon pool cleaning.

Methods

Sample Collection

Samples of *E. carteri* (EC) (Figure 1B) and *O. asiatica* (SS) (Figure 1C) were collected freshly (in triplicates and coded as EC1, EC2, EC3; SS1, SS2, SS3) from the Kaliporong in Surabaya, East Java (Figure 1A). Sponges were collected during the river's lowest tide (approximately 20–30 cm depth) using hand collection. The six specimens were immediately placed in sterile containers and temporarily frozen prior to microbiome extraction.

Microbiome Isolation

Microbiome was extracted using the SDS-based DNA extraction method (Vesty et al. 2017). A total of 0.25 g of frozen sponges were homogenized by mechanical grinding with the addition of 500 µL of DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1 % (wv⁻¹) cetyltrimethylammonium bromide (CTAB)], and 20 µL of proteinase K (10 mg ml⁻¹) (Vesty et al. 2017). Furthermore, each sample of sponges (EC1, EC2, EC3; SS1, SS2, SS3) was moved to microtubes and shaken at 225 rpm for 30 min at 37 °C temperature. SDS was added to each microtube, then incubated at 65 °C for 2 h with gentle inverting every 20 min.

Samples were then centrifuged at 6000×g for 10 min. The supernatants were collected in the new microtubes and added an equal volume of chloroform : isoamyl alcohol (24 : 1, v v⁻¹). The mixtures were centrifuged at

6000×g for 5min and then the upper phase was recovered in the new micro-tubes. Isopropanol was added to 60 % of the total mixture volume. The mixture was then incubated at room temperature for 1 hour and subsequently centrifuged at 14,000×g for 20 min. The DNA pellet was washed using 70 % (v v⁻¹) cold ethanol and resuspended in distilled water. Finally, all DNA extracts were purified using MO-BIO PowerClean® DNA Clean-Up Kits and quantified using Qubit dsDNA HS Assay Kit, run on 1 % agarose gel in sodium borate buffer to check the quality of DNA.

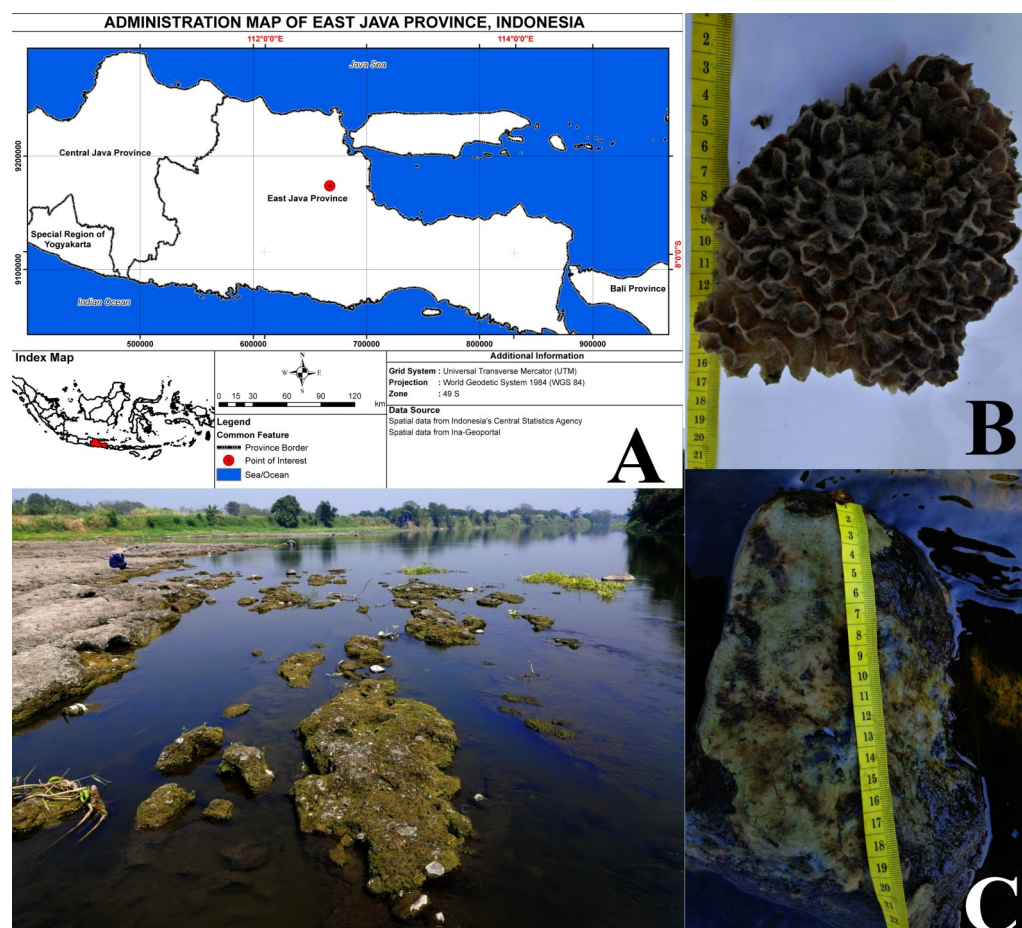


Figure 1. Sampling site and freshwater sponges: a. *Eunapius carteri*; b. *Oncosclera asiatica*.

16S rRNA Sequencing

16S rRNA rRNA gene region (V4) were amplified using specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'-GGACTACHVHHHTWTCTAAT) with the barcode (Walters et al. 2016). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The cycling conditions of the PCR program were as follows: 3 min at 94 °C for activation, followed by 35 cycles of 94 °C for 45 s for denaturation, 50 °C for 60 s for annealing, and 72 °C for 90 s for elongation, with a final elongation cycle of 72 °C for 10 min and hold at 4 °C. The PCR amplicons were mixed in the same volume of 1X loading buffer (contained SYB green) and examined by 2 % TAE agarose gel electrophoresis run at 100 V for 30 min. Expected band size for 515F-806R is 400-450 bp. Low-biomass samples may yield faint or no visible bands. Amplicon with bright main strips were chosen for further experiments. The amplicon pool was cleaned using Mo-Bio UltraClean PCR Clean-Up Kit and the concentration was measured A260/A280 ratio should be between 1.8-2.0 for the best results of final pool. For Sequencing of the 16S rRNA amplicon was performed by the Illumina HiSeq platform to produce 250 bp paired end reads.

Data Analysis

First step, paired-end reads were merged using FLASH (V1.2.7). Chimera sequences or artifacts were detected and removed using UCHIME (Edgar 2016) with the Gold referenced database. The overall processed were performed by Qiime (Version 1.7.0). OUT clustering was performed by Uparse software with 97 % similarity. Next, the representative sequence of each OTU was assigned for its taxonomy against the SILVA SSUrRNA database (Yilmaz et al. 2014) by RDP classifier (Wang & Cole 2024). OTUs abundances were normalized to the sequencing depth of the sample with the fewest reads. Subsequent analyses of alpha and beta diversity were conducted using the normalized data. To compare the microbial diversity between two sponges, alpha diversity indices including observed OTUs, Shannon diversity index, chao1 estimator, and PD whole tree were calculated. In addition, beta diversity analysis was performed to evaluate differences of samples in species complexity using both weighted and unweighted UniFrac methods.

Weighted UniFrac incorporates taxon abundances to highlight microbial shift by dominant taxa, whereas unweighted UniFrac relies on presence-absence to detect changes in rare lineages. Principal Coordinate Analysis (PCoA) has been performed from UniFrac distance matrix and visualized via ggplot2 package in R software. The Adonis test (using the Adonis function from the Vegan package) was conducted to statistically test the difference in microbial profiles between *Eunapius carteri* and *Oncosclera asiatica*. To expand the scope of our study, we performed the functional gene prediction from 16S rRNA data by Picrust (detail in PICRUST2.pdf, Douglas et al. 2020). Moreover, The KEGG (Kyoto Encyclopedia of Genes and Genomes database) was used to analyze the biodegradation pathways that reflect on the predictive functional profiling.

RESULTS AND DISCUSSION

Results

Sequencing Results

We obtained in total 975,160 read ranging from 123,494 to 178,854 sequences from six samples. These consist of 503,072 reads from *E. carteri* ranging from 164,505 to 178,985 sequences (Sequence Read Archive/ SRA accession number SRX15600599, SRX15600600, SRX15600601) and 472,088 reads from *O. asiatica* ranging from 123, 494 to 178, 854 sequences (Sequence Read Archive/ SRA accession number SRX15600602, SRX15600603, 15600604). After trimming in QC process for avoiding chimeras and singletons, 739,774 reads that consist of 372,610 sequences from *E. carteri* and 367,164 sequences from *O. asiatica* were yielded.

In addition to alpha diversity, we evaluated microbial community structure using Adonis (PERMANOVA) and Welch t-tests. The Adonis Unweighted UniFrac results yielded a pseudo-F value of 2.039 with a p-value of 0.107, while Adonis Weighted UniFrac analysis resulted in a pseudo-F value of 3.052 and a p-value of 0.088. Although these p-values were above the typical threshold of statistical significance ($p < 0.05$), they suggest a trend toward distinct colony compositions between the two sponge species, particularly when microbial abundance is taken into account.

Welch's t-tests further supported differences in colony richness and structure. Statistically significant differences were observed in Shannon diversity ($p = 0.019$), observed OTUs ($p = 0.040$), Chao1 richness estimator ($p = 0.042$), and Faith's phylogenetic diversity (PD Whole Tree; $p = 0.050$). These results indicate that *E. carteri* samples consistently host a more phylogenetically rich and diverse bacterial colonies than *O. asiatica*. While beta diversity metrics indicate compositional differences, these alpha diversity tests underscore the significantly richer microbial diversity in the *E. carteri* sample.

Alpha and Beta Diversity of Microbial Communities

Three samples of *E. carteri* showed values of 1138; 1030; and 624 OTUs, in contrast to the *O. asiatica* samples, which showed significantly lower values of 257; 221; and 470 OTUs—approximately one-third as many. Similarly, Shannon diversity index values for *E. carteri* were higher at 3.82, 3.51, and 4.12 compared to *O. asiatica*, which were 0.49, 0.42, and 1.93. The higher microbial diversity of *E. carteri* was further supported by chao1 richness estimates at 1210, 1114, and 700, compared to *O. asiatica*, which showed lower values of 298, 353, and 536. Similarly, PD whole tree values were higher in *E. carteri* (118, 89, and 76) than on *O. asiatica* (52, 43, and 41). Although the average alpha diversity indices of *E. carteri* are higher than those of *O. asiatica*, the results from the Welch's t-test are not significantly different due to the low number of replicates (Figure 2).

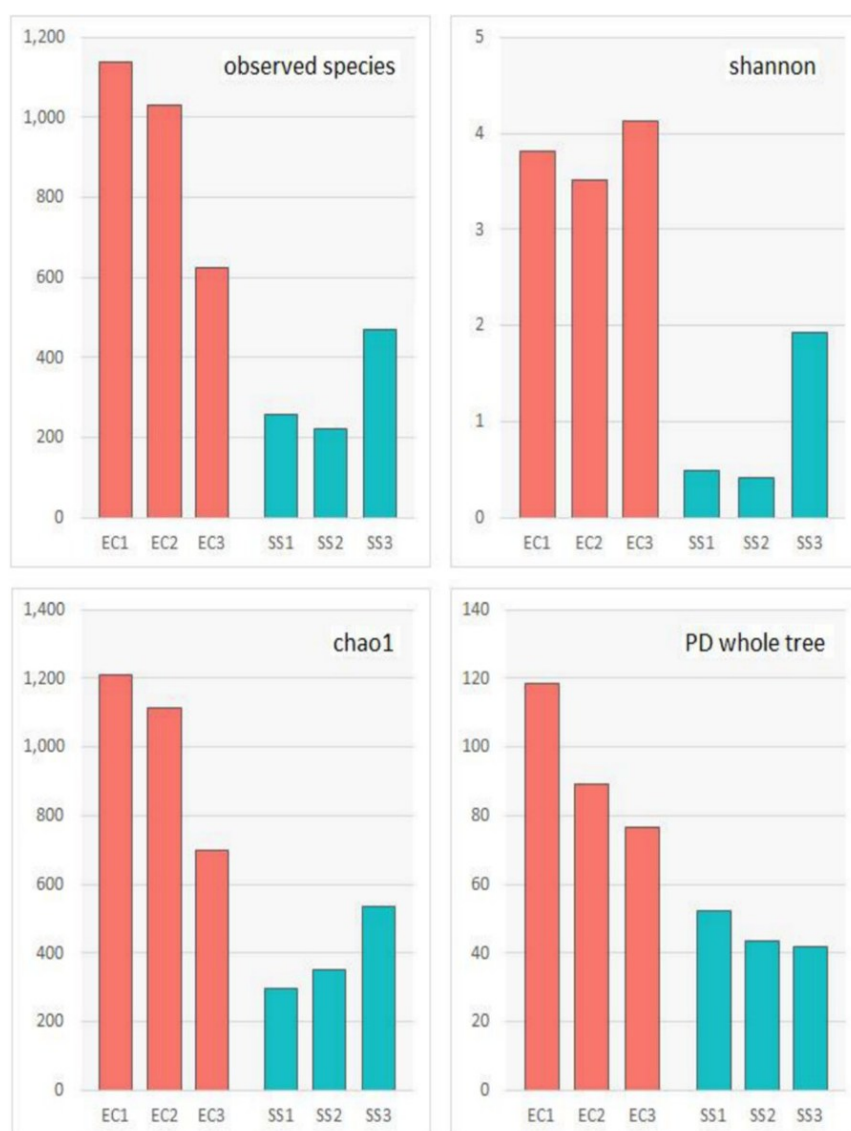


Figure 2. Comparison of alpha diversity index across samples. Four alpha diversity metrics; (A) observed OTUs, (B) Shannon diversity index, (C) Chao1 richness estimator, and (D) Faith's phylogenetic diversity (PD whole tree), computed for each of six samples (EC1–EC3 and SS1–SS3), with the x-axis representing individual sample IDs, and the y-axis showing the diversity value for each metric.

The microbiome composition of symbiont from *O. asiatica* samples is more uniform than *E. carteri*. This was further observed by beta diversity displayed on principal coordinate analysis (PCoA) plot (Figure 3). The assessment of species richness using rarefaction plot, based on the sampling results, indicates sufficient sampling depth. This is evidenced by the sampled sequence-

es from *O. asiatica*, which show greater saturation compared to *E. carteri* (Figure 4). Moreover, unique OTUs from *E. carteri* are higher than *O. asiatica* samples, reflecting more diverse and unique symbionts of *E. carteri* than *O. asiatica* (Figure 5, Table 1).

The Principal Coordinates Analysis (PCoA) plots illustrate the beta diversity of microbial colonies in freshwater sponges *E. carteri* and *O. asiatica*. PCoA plot based on the weighted UniFrac distances (left) indicates clear separation between the microbial communities of SS and EC in the first principal component (PC1), which explains 80.43 % of the variation. Meanwhile, PCoA plot based on the unweighted UniFrac distances (right), which consider only the presence or absence of taxa, also indicating distinct clustering between SS and EC groups along PC1, accounting for 35.39 % of the variation. Both plots demonstrate compositional differences between the microbial colonies in the two sponge groups observed in this study.

The assessment of species richness using rarefaction curves (Figure 4) confirmed the sufficient sequencing depth in both sponge species. However, the *E. carteri* samples showed steeper curves and a broader range of observed OTUs, indicating a richer and more diverse microbial community than *O. asiatica*. The plateauing of rarefaction curves across all samples indicates a mini-

Table 1. Number of detected OTU based on cleaned sequences among selected samples of freshwater sponges (EC= *E. carteri*; SS= *O. asiatica*) and the diversity indexes.

Summary of Sequencing Data and Alpha Diversity Index									
Sample ID	No. Raw Read	No. Cleaned Read	Observed Species	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole Tree
EC1	176.985	118.366	1.138	3,815	0.793	1.211	1.229	0.998	118,615
EC2	161.582	124.926	1.03	3,507	0.689	1.114	1.149	0.998	89,366
EC3	164.505	129.318	624	4,124	0.879	701	732	0.999	76,609
SS1	169.74	125.43	257	0,486	0.091	299	308	0.999	52,432
SS2	123.494	94.618	221	0,422	0.078	353	328	0.999	43,393
SS3	178.854	147.116	470	1,93	0.38	536	522	0.999	41,696
Adonis Unweighted Unifrac									
Group 1	Group 2	Sample size	Permutations		pseudo-F		p-value	q-value	
EC	SS	6	999		2.038758189		0.107	0.107	
Adonis Weighted Unifrac									
Group 1	Group 2	Sample size	Permutations		pseudo-F		p-value	q-value	
EC	SS	6	999		3.051691559		0.088	0.088	
Welch t-test result EC vs SS									
feature		t_statistic			p_value		p_adj		
shannon		5.480285			0.01866		0.050368151		
observed_OTUs		3.518411			0.040448		0.050368151		
chao1		3.559903			0.041973		0.050368151		
PD_whole_tree		3.808369			0.050478		0.050477551		

mal gain in new OTUs with increasing sequencing depth, suggesting that most microbial diversity was adequately captured.

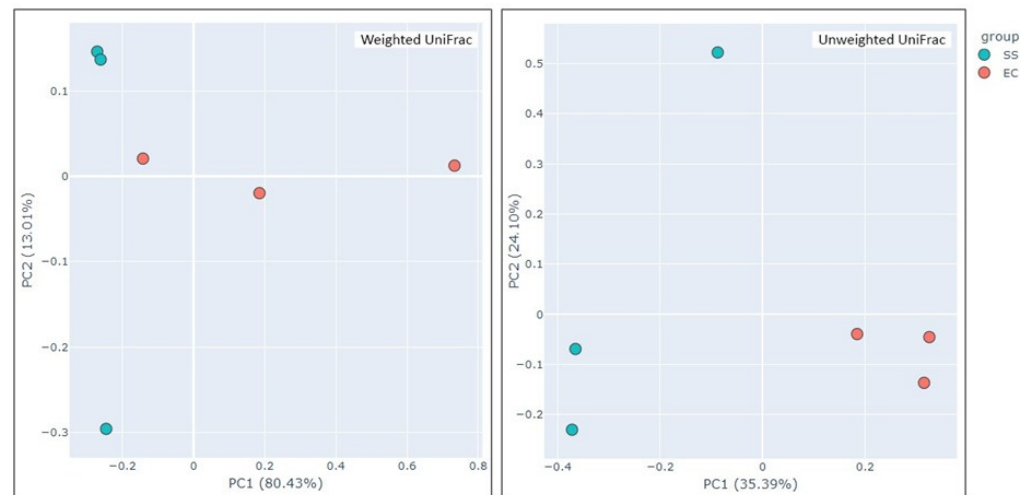


Figure 3. Beta Diversity Analysis Using Weighted and Unweighted UniFrac Metrics.

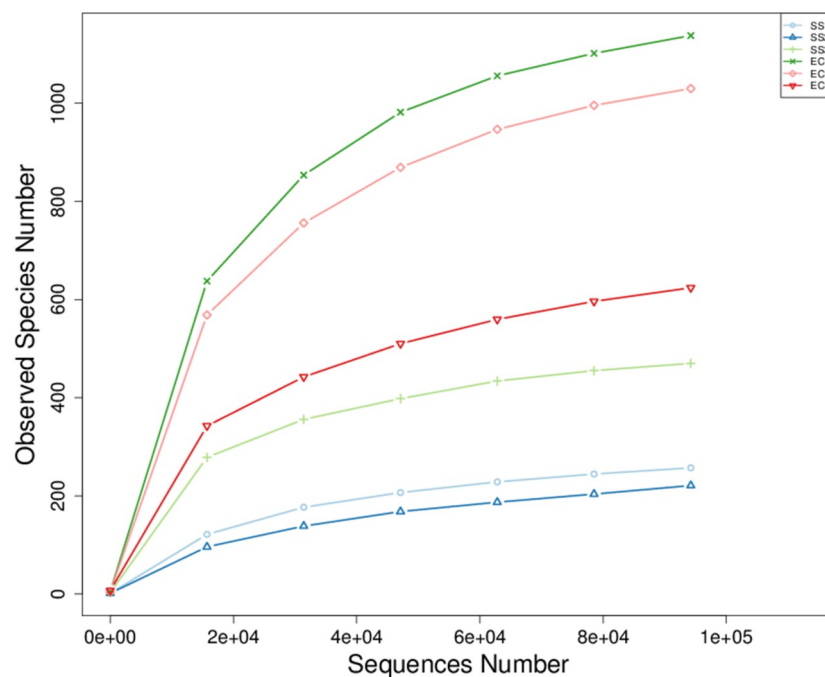


Figure 4. Rarefaction curves showing observed species richness of microbial colonies in *E. carteri* and *O. asiatica*.

Further, the Venn diagram (Figure 5) highlights the shared and unique in microbial colonies among the samples. *E. carteri* displayed more unique OTUs than *O. asiatica*, supporting earlier studies suggesting it harbors a more diverse and distinct microbiome. The shared OTUs between both sponge species likely represent a core microbial colony characteristic of the Kaliporong River environment, while the unique OTUs underline species-specific bacterial associations. These figures collectively reinforce the conclusion that *E. carteri* harbors a broader and more exclusive bacterial diversity than *O. asiatica*.

Our study revealed higher values of taxonomic richness of microbial colony, highlighting biogeographic patterns at the family/genus/species levels from two freshwater sponges *E. carteri* (Bowerbank, 1863) and *O. asiatica*

(Manconi & Ruengsawang, 2012) in Kaliporong river, Surabaya, East Java, Indonesia. Recent findings of previously undocumented freshwater sponge species and their associated microbial communities in Indonesia suggest that their biodiversity is still largely underexplored. Further investigations should explore the potential bioactive compounds produced by freshwater symbionts, which may contribute to the development of new antibiotics or antiviral agents.

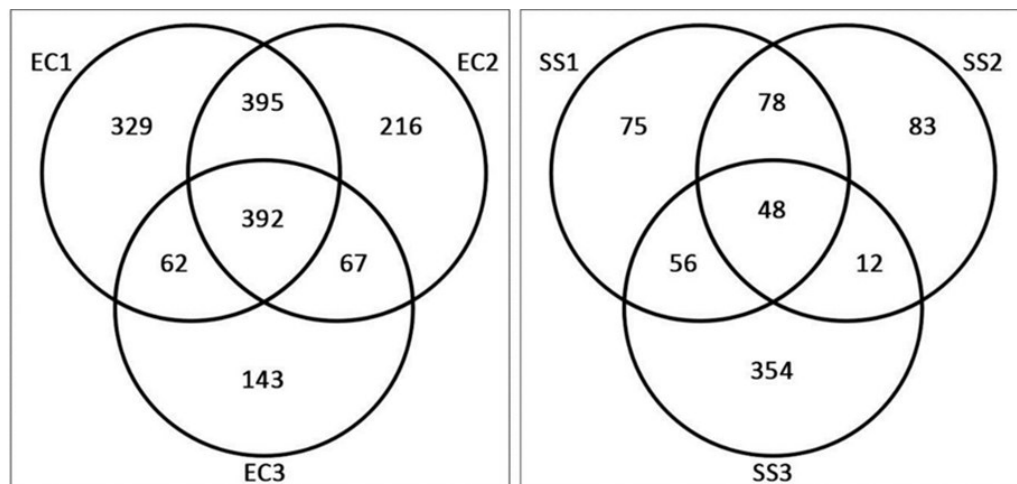


Figure 5. Venn diagram shows the number of shared and unique OTUs of microbial colonies in *E. carteri* and *O. asiatica*.

Bacterial Composition Profile (Phylum/Class and Genus)

We identified 28 bacteria phyla in the freshwater sponges under study with the majority being Proteobacteria phylum in both *E. carteri* (50 %) and *O. asiatica* (90 %). Although Proteobacteria dominated the symbiotic communities in both sponge species, each hosted a distinct bacterial phylum composition. In addition to Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria were also detected in both species. Nevertheless, *E. carteri* harbors a larger population of Cyanobacteria and unknown bacteria group, whereas *O. asiatica* shows a higher relative abundance of Firmicutes and Bacteroidetes (Figure 6). This figure showed the comparison of bacterial profiles between EC and SS samples. Furthermore, *Aeromonas*, *Pseudomonas*, and unclassified genera were dominant in *E. carteri*, whereas *O. asiatica* exhibited a higher prevalence of *Pseudomonas* and *Ralstonia* (Figure 7).

Predictive Functional Profiling

Xenobiotic degradation and metabolism accounted for 0.96 of the total predicted functions in *E. carteri* and 1.12 % in *O. asiatica*, based on the 52 second-level pathway categories from the KEGG Orthology (KO) database (Figure 8). Given the large number of KOs associated with xenobiotic degradation and metabolism pathways in the KEGG database, the top ten KOs were selected for further analysis, including K01692, K00626, K01825, K00632, K01782, K00121, K00128, K01951, K00088, and K00799. The curation of these top KO numbers is related to major xenobiotic degradation and metabolism pathways, such as the pathway of xenobiotic and drug metabolism by cytochrome P450, degradation of benzoate, degradation of caprolactam, and degradation of aminobenzoate (Figure 9). Xenobiotic metabolism by cytochrome P450 had the highest relative abundance among the selected pathways, with at least seven associated genes or enzymes identified (E1.1.1.1, CBR1, frmA, ALDH3, GST, EPHX1, HPGDS). This is followed by drug metabolism by cytochrome P450 and by other enzymes. In general, the relative abundance of degradation pathways present in *E. carteri* samples was lower

than *O. asiatica* samples. Notably, benzoate degradation, another pathway with relatively high abundance, was represented at 5.03 % in *E. carteri* and 5.81 % in *O. asiatica*, involving at least 60 associated genes or enzymes.

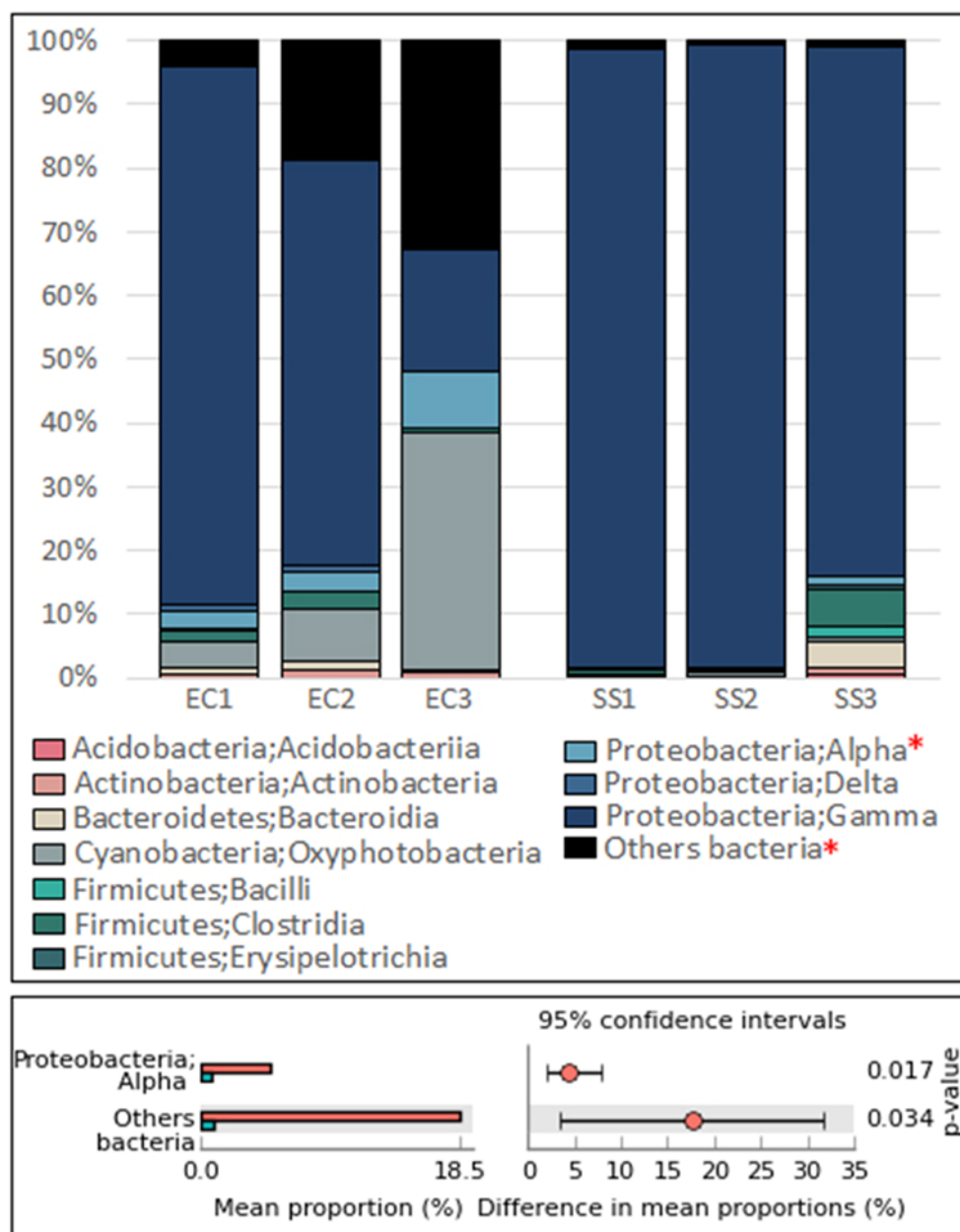


Figure 6. Comparison of relative abundance phyla in *E. carteri* and *O. asiatica*.

Discussion

The estimated bacterial symbiont diversity in our *E. carteri* samples, with 996 observed OTUs, aligns closely with findings from *E. carteri* in India, which reported 980 OTUs (Gaikwad et al. 2016). However, different composition of symbiotic bacteria in those two different locations were detected, since in India, Firmicutes constituted the highest proportion, followed by Cyanobacteria and Actinobacteria. By contrasts, our *E. carteri* harbors the largest composition of Proteobacteria, followed by Cyanobacteria and unidentified bacteria. This difference may be attributed to variations in habitat or environmental conditions that influence the composition of symbiotic bacteria (Weigel & Erwin 2016; see detail in Hill & Sacristán-Soriano 2017; Ferreira et al. 2020). Furthermore, the approximately one-third lower OTU count in *O. asiatica* (335) might be due to differences in sponge morphology. *E. carteri* exhibits more encrusting, lobate, and arborescent forms, whereas *O. asiatica* has a massive,

thinner structure and exclusively attaches to rock substrates. Consistent with the influence of morphology, other Spongillids with encrusting and lobate forms have also been shown to harbor significantly higher OTU diversity of symbiotic bacteria in *Corvospongilla lapido* (2900 OTU) (Gaikwad et al. 2016) and *Trochospongilla pennsylvanica* 2 (approximately 1800 OTU) (Laport et al. 2019). Moreover, gradual colonization, functional redundancy of symbiotic microbe and more specifically, water exposure on host, also influence microbe diversity and its OTU number (Webster & Thomas 2016), as sponges obtain nourishment by filtering water from the surroundings.



Figure 7. Heatmap of relative abundance of microbial profile at the genus-level. The heatmap shows the percent relative abundance of bacterial genera across six samples, similarly clustered as shown by the dendrogram.

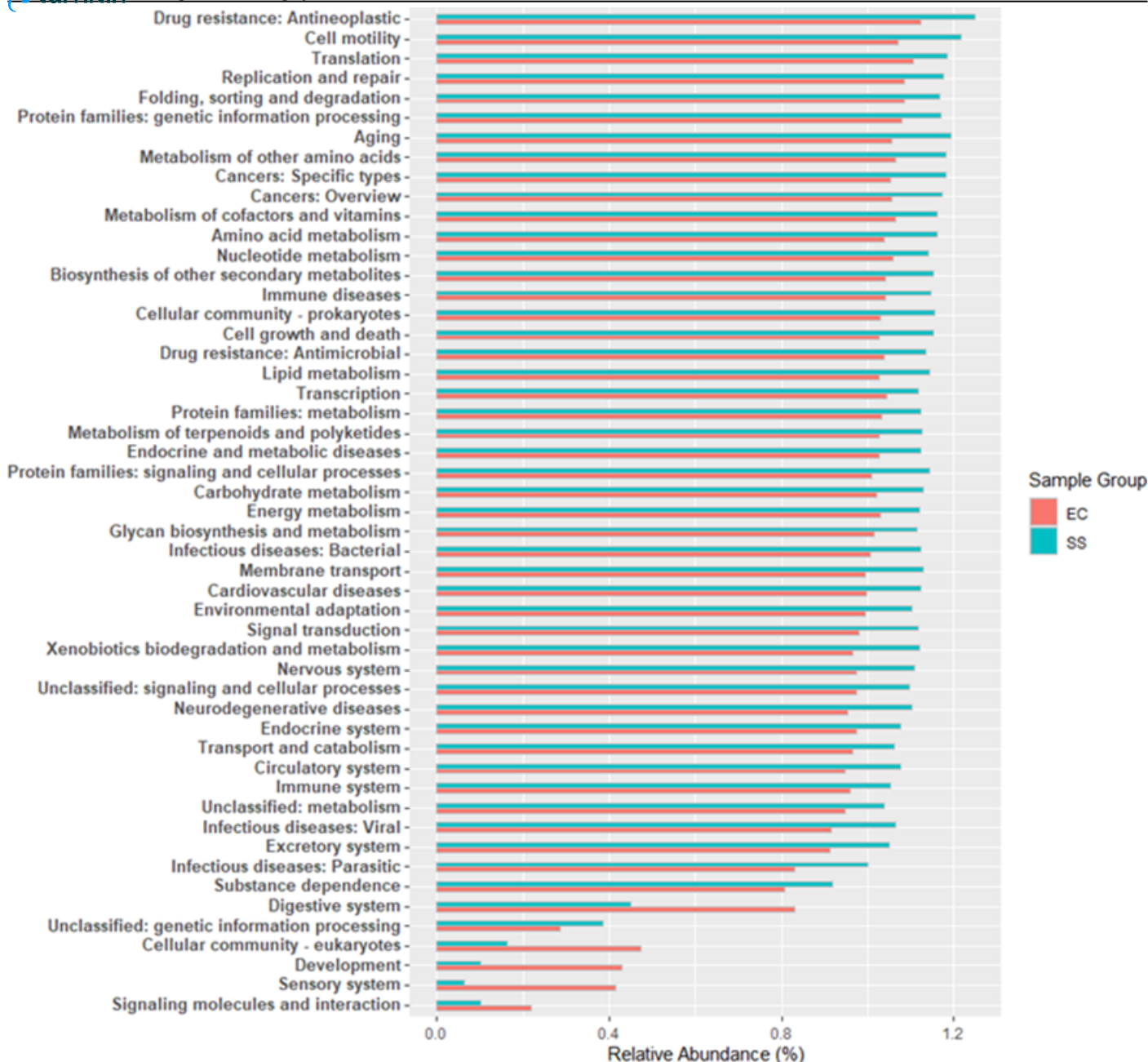


Figure 8. Predicted second-level pathways in KEGG for each group of *E. carteri* and *O. asiatica*.

Domination of gammaproteo bacterial on both of our species aligns with findings on symbionts of the spongillid *Ephydatia fluviatilis* (Keller-Costa et al. 2014). Gammaproteo bacteria has been recognized as one of the major components of marine, intertidal and freshwater environment (Francis et al. 2021). Besides possessing a vital role on carbon, nitrogen, and sulfur cycling (Nikrad et al. 2014; Baker et al. 2015), this microbial classis is also known for its medicinal, industrial, and environmental properties. The biggest genus of symbiont *O. asiatica* (Pseudomonas) plays an important role in antibiotic production, waste degradation, enzyme, and pigment and biosurfactant production, i.e., *P. aeruginosa* (Anayo et al. 2019). Likewise, the second biggest symbiont of *O. asiatica*, genus Ralstonia also plays an important role in biomaterial production like acrylamide (Aisami & Gusmanizar 2019) and poly [hydroxyalkanoate] (Priyadarshi et al. 2014).

In contrast to *O. asiatica*, the genus Aeromonas from gammaproteobacterial and unidentified bacterial phylum dominates *E. carteri*. Aeromonas is known for its important role in regulating the population of copepods (Gugliandolo et al. 2008; Chaix et al. 2017), which is an important food source

on freshwater food chain and ecosystem. Photosynthetic bacteria from Phylum Cyanobacteria represent the third major group of symbionts in our *E. carteri*, supporting previous findings that identified that Cyanobacteria as major components in *E. carteri* in India (Gaikwad et al. 2016). In addition to their marine counterparts, freshwater sponges also rely on Cyanobacteria for nitrogen fixation and protection against UV radiations (Kenny et al. 2019; Hall et al. 2021).

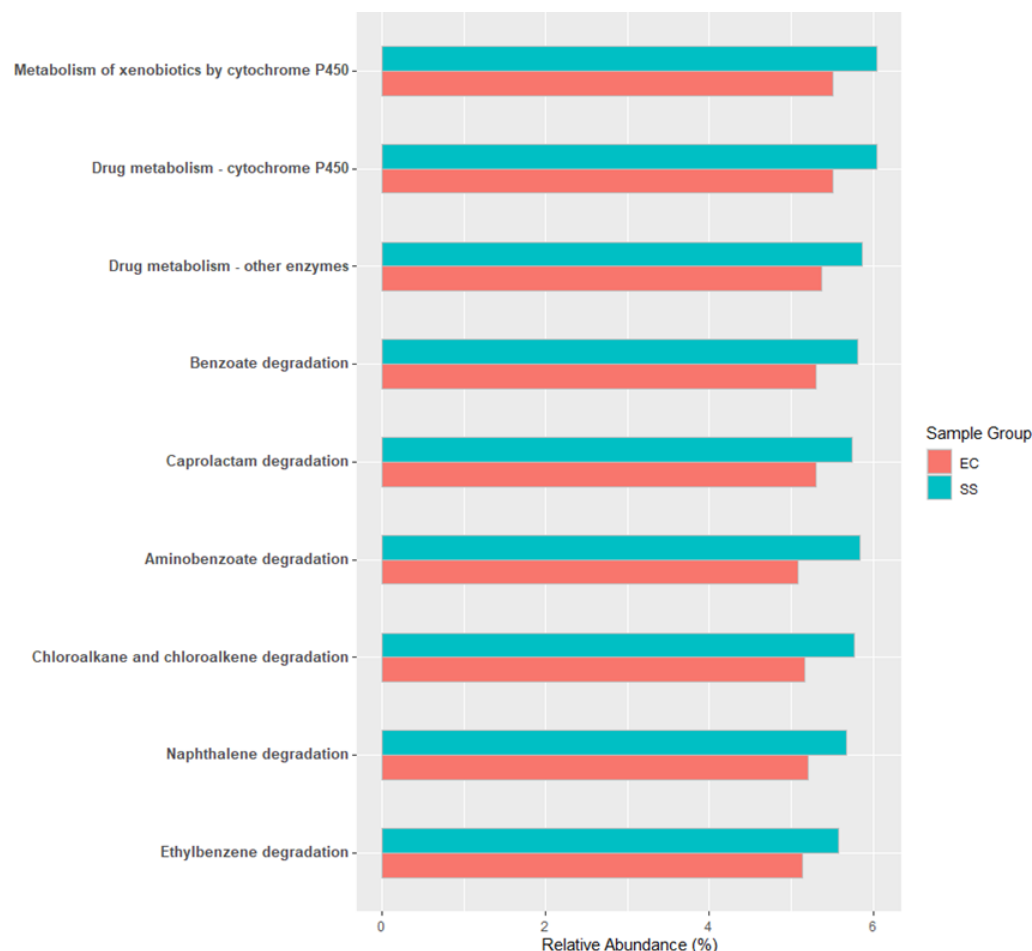


Figure 9. Predicted xenobiotic degradation pathways in KEGG for each group of *E. carteri* and *O. asiatica*.

Functional prediction analysis was performed to investigate the functional pathways in different freshwater sponges. Among the predicted functions, xenobiotic and drug metabolism via cytochrome P450 showed the highest relative abundance in both *O. asiatica* and *E. carteri* samples. Cytochrome P450 enzymes are known to play key roles in xenobiotic degradation and are also linked to major secondary metabolite pathways (Senate et al. 2019; Kunakom et al. 2023). Although *O. asiatica* has lower diversity than *E. carteri*, *O. asiatica* has a higher xenobiotic degradation capability due to the high abundance of the genus *Pseudomonas* and other contributing genus. Many bacteria do not contain cytochrome P450 (Kelly & Kelly 2013). However, there are some groups of bacteria such as *Pseudomonas putida* that can perform oxidative dehalogenation with a P450CAM intermediation (Greule et al. 2018). Other *Pseudomonas*, such as *P. aeruginosa* CYP168A1 was characterized as a fatty acid hydroxylase (Tooker et al. 2022). *Ralstonia metallidurans* also reported with one-component P450 system, expressing phthalate dioxygenase reductase (Alviz-Gazitua et al. 2022). The presence of GST was related in the degradation of xenobiotics, carcinogens, antioxidant, and peroxides (Zhang et al. 2021).

Cyanobacteria have been reported to contain a high concentration of

intracellular GSH (Narainsamy et al. 2016; Allocati et al. 2018; Rai et al. 2021; Cassier-Chauvat et al. 2023). This might explain the high amount of P450 degradation system in *E. carteri*. In addition, benzoate degradation was another pathway with relatively high abundance. Benzoate is an aromatic ester, an intermediate in the biodegradation of complex aromatic compounds such as toluene; and a precursor of catechol and protocatechuate, and also commonly used as model compounds to study bacterial metabolism of aromatic compounds (Muñoz-García et al. 2019; Yadav et al. 2021). Prediction results show that key genes such as *benA-xylX*, *benB-xylY*, *benC-xylZ*, and *benD-xylL* convert benzoate to catechol. Other genes, such as *catA*, *catB*, *catC*, *pcaB*, *pcaC*, *pcaD*, *pcaI*, and *pcaF* confirm the degradation of benzoate through the catechol-ortho cleavage pathway (Yadav et al. 2021).

Several studies (Nanjani et al. 2022) have reported that *Pseudomonas*, specifically *P. aeruginosa* D6 and *P. putida* P8 can degrade textile dyes from the benzoate group via both ortho- and meta-cleavage pathways. Aminobenzoate degradation is also closely related to benzoate degradation. The *abmG* and *antA* genes detected indicate the presence of both aerobic and anaerobic degradation of 2- Aminobenzoate (anthranilate). The *antABC* gene encodes an anthranilate dioxygenase enzyme that converts anthranilate to catechol, which is further degraded via ortho cleavage or meta cleavage pathways. The *abmG* gene encodes 2-Aminobenzoate-CoA ligase that converts 2-Aminobenzoate to 2-Amino-benzoyl-CoA. 2-Amino-benzoyl-CoA is converted into Benzoyl-CoA, which ultimately enters the benzoate degradation pathway (Arora 2015). The enrichment of xenobiotic degradation pathways, particularly in *O. asiatica*, suggests that this species and its microbiome may contribute significantly to detoxifying polluted river systems. This functional potential aligns with the detection of abundant cytochrome P450-related pathways and benzoate degradation genes. Taken together, these findings highlight the promise of *O. asiatica* as a valuable natural resource for freshwater bioremediation, especially in environments facing anthropogenic contamination.

Our study revealed higher values of taxonomic richness of microbial community, highlighting biogeographic patterns at the family/genus/species levels from two freshwater sponges *E. carteri* (Bowerbank, 1863) and *O. asiatica* (Manconi & Ruengsawang, 2012) from Kaliporong river in near Surabaya, East Java province Indonesia.

This research has shown that biodiversity of freshwater sponges has been underexplored, as indicated in this new record of species of freshwater sponges and their microbial community. Further investigations should explore the potential bioactive compound from the freshwater symbionts, which could contribute to the fulfilment of the demand for antibiotics or antiviral agents.

CONCLUSION

Our analysis of 16S rRNA gene sequences enabled the identification of major taxonomic groups and the estimation of bacterial symbiont diversity associated with selected freshwater sponges *E. carteri* and *O. asiatica*. These data enhance our knowledge of freshwater sponge microbial colonies in freshwater sponges, which are reliable indicators of the state of their aquatic environment.

AUTHORS CONTRIBUTION

E.S., C.R., A.B. designed the research. A.Y., L.M.J., F.P. collected the data and did the Laboratory works. W.M. analyzed the bioinformatic data. D.W., E.S., A.B. wrote the manuscript and supervised all the process.

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CONFLICT OF INTEREST

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

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