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High-throughput analysis using 16S rRNA sequencing of bacterial communities associated in selected mangrove species from Bayug Island, Iligan City, Philippines

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Abstract. *Siblos SKV, Tabugo SR. 2024. High-throughput analysis using 16S rRNA sequencing of bacterial communities associated in selected mangrove species from Bayug Island, Iligan City, Philippines. Biodiversitas 25: 53-61.* Mangrove ecosystems are recognized globally as highly productive ecosystems, that play a crucial role in carbon sequestration, erosion control, water purification, and as essential breeding grounds for diverse aquatic life. They also offer specialized ecological niches, accommodating a diverse range of organisms, including bacterial communities. This study aimed to investigate bacterial communities of Bayug Island in Iligan City, Philippines, inhabiting six mangrove species, namely *Rhizophora stylosa* Griffith, *Rhizophora apiculata* Blume, *Rhizophora mucronata* Lam., *Sonneratia alba* Sm., *Ceriops tagal* (Perr.) C.B.Rob., and *Nypa fruticans* Wurmb and their possible functions within the mangrove forests. Genomic DNA was extracted from the six pooled soil samples, and the V3-V4 region of the 16S rRNA gene was sequenced using the Illumina MiSeq platform. Six amplicon libraries, corresponding to the mangrove species were analyzed with Parallel Meta Suite software, yielding 173,270 amplicon sequence variants (ASVs) after quality control. The study identified the top five most abundant ASVs in the mangrove rhizosphere, linked to the genera *Vibrio*, *Stenotrophomonas*, *Serratia*, *Pseudoalteromonas*, and *Achromobacter*. Among the mangrove species, *S. alba* exhibited higher alpha diversity according to the Shannon index. PICRUSt analysis revealed that microorganisms are involved in biodegradation, xenobiotic metabolism, and other metabolic processes within the mangroves. These bacteria could find applications in environmental cleanup, bioremediation, waste treatment, and soil health improvement. This result contributes to the ongoing ecological restoration of mangrove forests at the research site.

Keywords: Abundance, bacterial communities, Bayug Island, diversity, mangrove species

INTRODUCTION

Mangroves are recognized globally as highly productive ecosystems (Mai et al. 2021), that play a crucial role in carbon sequestration, erosion control, and water purification (Akram et al. 2023). They are vital nurseries for marine life and effective debris traps (Abreo et al. 2020). Despite their ecological significance, mangrove forests are increasingly threatened by human activities like deforestation, pollution, and land reclamation (Agduma and Cao 2023). Thriving in intertidal zones of tropical and subtropical coastlines (Pillai and Harilal 2018), mangroves feature species adapted to diverse environmental conditions shaped by geography and hydrology (Gómez-Acata et al. 2023). Their ability to survive in intertidal zones is associated with microorganisms in the sediment, positioning them as focal points for microbial diversity (Bai et al. 2013; Haldar and Nazareth 2018; Chaudhuri et al. 2019).

The microbiota within mangrove ecosystems comprises a combination of terrestrial, marine, and freshwater organisms, all playing crucial roles in sustaining and regulating the functions of the mangrove environment (Liu et al. 2019). Microorganisms in mangrove sediments actively engage in biogeochemical cycles and serve as a primary nutritional source for plants and animals in these ecosystems (Mendes and Tsai 2014). According to Muwawa et al. (2021), there is a close and mutually beneficial relationship between mangrove species and microorganisms in Kenya based on 16S rRNA metagenomic analysis. Certain microorganisms, like nitrogen-fixing, phosphate-solubilizing, and sulfate-reducing bacteria, isolated from mangrove soil, positively impact mangrove growth (Sukmawati et al. 2022). In return, mangroves provide a habitat for these microbes to thrive and provide essential nutrients to support their growth (Yulma et al. 2020). This interplay highlights the importance of microorganisms in mangrove forests for boosting productivity, conservation, and ecosystem restoration (Erazo and Bowman 2021).

Traditional approaches to studying bacterial communities have historically relied on microscopic methods for identification and quantification (Yu et al. 2015). Nevertheless, these techniques have limitations, because of the small size of microorganisms and their lack of well-defined taxonomic characteristics, making accurate microscopic identification challenging (Navgire et al. 2022). In recent years, Next-Generation Sequencing (NGS)

has become a powerful tool for studying soil microorganisms, including diazotrophic communities (Shiau et al. 2021). In particular, ribosomal RNA (rRNA) gene sequencing is widely accepted for assessing bacterial diversity (Zhou et al. 2017). Illumina MiSeq sequencing allows swift and cost-effective sequencing of diverse nucleic acids, enabling the simultaneous sequencing of millions of DNA fragments in a single operation (Singh and Kumar 2021). NGS has various applications, spanning whole genome sequencing, RNA-seq, metagenomics, and epigenomics, advancing our understanding of genetics and molecular biology (Satam et al. 2023). The Illumina MiSeq platform, commonly used in microbial ecology research, is well-suited for various applications like genome sequencing, metagenomics, amplicon sequencing, and targeted sequencing of specific genomic regions. (Nathan et al. 2020). Both methods have been proven effective for monitoring changes in bacterial and diazotroph communities within marine ecosystems like mangroves (Decembrini et al. 2021).

There is a dearth of comprehensive studies identifying bacteria concerning specific mangrove species, making this research endeavor particularly important (Mai et al. 2021). This study aims to elucidate the abundance and diversity of microbial communities across different mangrove hosts, explore their functional roles, and investigate potential connections between mangrove hosts and microbial diversity. It is essential to investigate whether distinct microbial compositions are associated with various mangrove species or hosts and to ascertain the existence of a common core microbial community shared among mangroves. With this, the protection and rehabilitation of the study area will be strengthened for the restoration of the once-flourishing mangrove area on the island of Bayug, Iligan City, Philippines.

MATERIALS AND METHODS

Study site

Sampling was conducted in Bayug Island Sitio of Barangay Hinaplanon 3.7 km NE of población in Iligan City, Philippines. The study site is the reforested mangrove areas in Iligan Bay. It lies geographically 8°15'30" N and 124°14'56" E (Figure 1).

Procedures

Collection of samples

The coast of Bayug Island was once lined by vast mangrove forests. However, due to natural disasters and erosion, the once-flourishing mangrove forest was destroyed. Now, a chunk of the island has been swallowed by the sea from its original area of 122.50 ha. It has shrunk to 82 ha, which continues to advance inland (Almarez et al. 2014). Despite all this, the efforts of the locals and the Mindanao State University-Iligan Institute of Technology (MSU-IIT), a rehabilitation program was established in the study area until today.

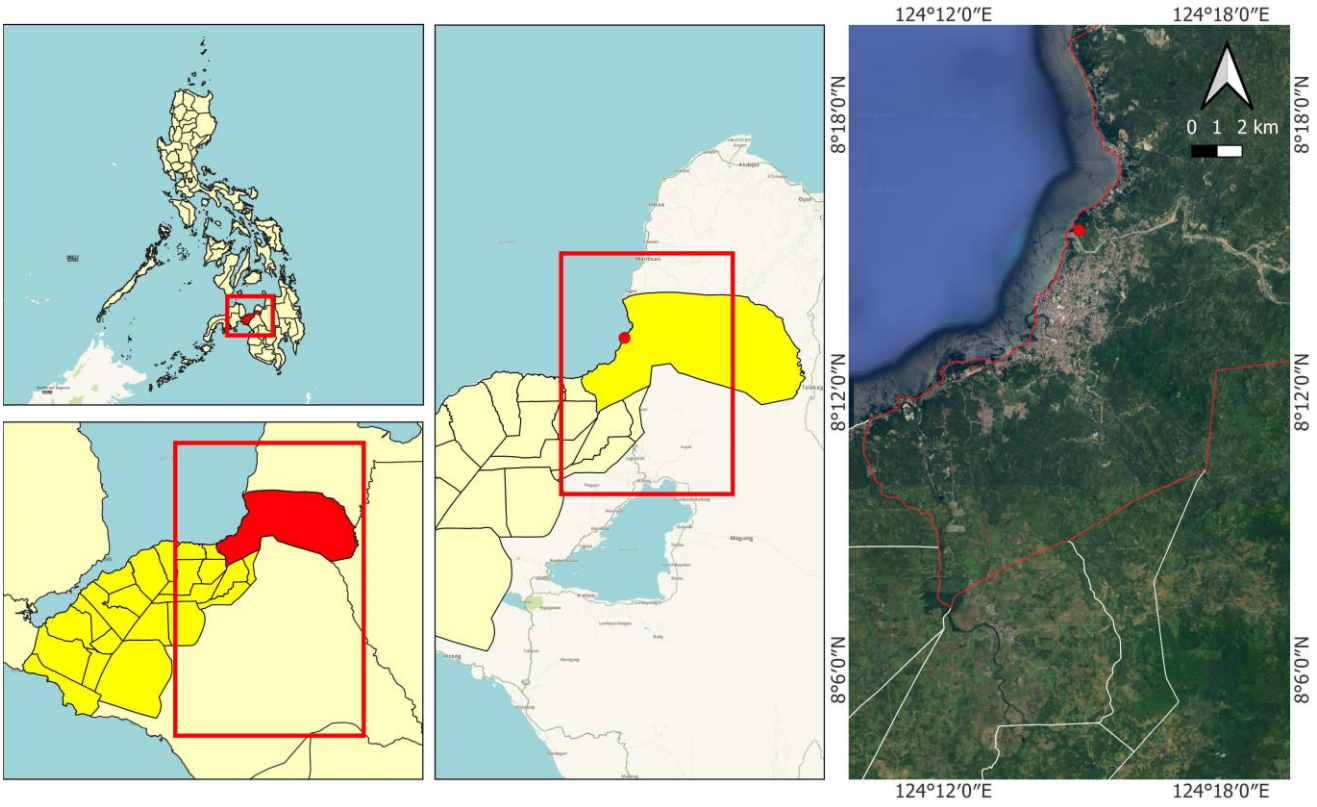

Figure 1. Map of the study area: Mangrove area of Bayug, Island, Iligan City, Philippines

Sediment samples were gathered from Bayug Island in Iligan City, Philippines. Home of at least 4-6 mangrove species, namely *Rhizophora apiculata* Griffith, *Rhizophora stylosa* Blume, *Rhizophora mucronata* Lam., *Sonneratia alba* Sm., *Ceriops tagal* (Perr.) C.B.Rob., and *Nypa fruticans* Wurmb. These mangrove species, approximately 1.5 m in height, were included in the study. Within a proximity of 1 to 10 m from each other, three individual plants of each species were selected for investigation. Sediment samples were collected from their rhizosphere. Sampling involved vertically retrieving rhizosphere sediments along the base of each plant using a core sampler. The collected sediments from the rhizosphere were obtained from two distinct depths: 1-5 cm and 10-15 cm below the surface. Subsequently, the rhizosphere soil underwent a sieving process using a 2 mm sieve to ensure homogenization and to remove residual roots and debris. Triplicate samples for each mangrove species were meticulously blended to create a comprehensive composite sample for further analysis. These composite samples were then placed in sterile plastic bags and stored in an icebox for transportation to the laboratory. Upon arrival at the laboratory, they were preserved at -20℃ to facilitate DNA extraction (Muwawa et al. 2021).

Measurement of physico-chemical parameters

Soil parameters, which include temperature, pH, and salinity, were recorded in situ. The sample processing and analysis were conducted at the Molecular Systematics and Conservation Genomics Laboratory, part of the Center for Biodiversity Studies and Conservation (CBSC) within the Premier Research Institute of Science and Mathematics (PRISM), Mindanao State University -Iligan Institute of Technology, Iligan City, Philippines.

DNA extraction, amplification, and MiSeq sequencing

The extraction of total genomic DNA (gDNA) from the collected samples was carried out individually using the HiPurA® Soil DNA Purification kit (Himedia Laboratories, India), following the manufacturer's instructions. The quality of the extracted gDNA was assessed using gel electrophoresis on Certified Molecular Biology Agarose gel (BIO-RAD) in 1x TBE buffer, employing the Cleaver Scientific electrophoresis system (MSMINIONE). GelGreen dye (CA, USA) at a concentration of 10,000x in water was used to stain the gels. Subsequently, the samples were sent to Macrogen, Korea, for amplification and metagenome custom amplicon sequencing after undergoing a quality check. The amplification of DNA was performed using the following primers: Forward Primer (Bakt_341F-long): AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCT CAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG. Reverse Primer (Bakt_805R-long): CAAGCAGAAGACGGCA TACGAGATGTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATCC (Muwawa et al. 2021).

The Polymerase Chain Reaction (PCR) procedure began with an initial denaturation step at 98℃ for 30 seconds, followed by 10 cycles consisting of denaturation at 98℃ for 10 seconds, annealing at 55℃ for 10 seconds, and extension at 72℃ for 30 seconds. Subsequently, an additional 25 cycles were carried out: denaturation at 98℃ for 10 seconds, annealing at 65˚C for 10 seconds, and extension at 72℃ for 30 seconds. The amplification process concluded with a final extension step at 72℃ for 2 minutes (Muwawa et al. 2021). After undergoing a quality check, six amplicon libraries were constructed for further analysis.

MiSeq utilizes Illumina's proprietary sequencing by synthesis technology. This involves the cyclic addition of fluorescently labeled nucleotides to growing DNA strands, followed by imaging to determine the sequence. MiSeq is frequently utilized for studying microbial communities through 16S rRNA gene sequencing. This study utilizes this platform for its flexibility and user-friendly interface, making it accessible to a broad range of researchers (Singh and Kumar 2021).

Data analysis

All data processing and study of MiSeq raw reads were conducted using the Parallel Meta Suite (PMS) pipeline. PMS is a highly efficient and interactive tool for microbiome data analysis, providing accuracy and speed. In this study, amplicon sequencing was employed. PMS initially identifies amplicon sequence variants (ASVs) and performs de-chimerization to ensure accuracy and includes diversity indices like Shannon index. The sequences are then aligned and compared against reference databases using the integrated vsearch tool, generating profiles and annotating taxonomy from the broad kingdom to the specific species level. The relative abundance of taxa, as well as alpha and beta diversity were determined, and gene families were inferred using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm. Metabolic pathways were also annotated using the KEGG BRITE hierarchy, which systematically examines gene functions by linking genomic data to more advanced functional insights (Chen et al. 2022).

RESULTS AND DISCUSSION

Physico-chemical parameters

Physico-chemical parameters were meticulously measured, revealing an average water temperature of approximately 28.4℃, a water pH level of around 7.6, and a salinity of 30 ppt. Studies indicate that the dynamic environment within mangrove ecosystems, shaped by regular tidal fluctuations, pH levels, temperature variations, salinity levels, light exposure, rainfall patterns, and nutrient availability, plays a critical role (Thatoi et al. 2013; Tavares et al. 2021). It is worth noting that bacteria play a pivotal role in enhancing the productivity, conservation, and rehabilitation of mangrove ecosystems. The health and ecological conditions of the mangrove forest significantly influence microbial communities (Liu et al. 2019). Planting more mangroves could potentially bolster bacterial communities in the area.

According to Gusman et al. (2022), the conditions of mangrove soil and water quality were conducive to *Vibrio* bacteria's growth. *Vibrio* bacteria were specifically detected in soils with a pH range of 6-7, a salinity of 30 ppt, and temperatures ranging from 28 to 37°C (Sampaio et al. 2022). This trend also extends to other bacteria inhabiting mangrove soil, which can thrive within pH levels from 5 to 7 and under a maximum temperature of 40°C (Behera et al. 2016). It has been observed that the genera *Rhizophora*, *Sonneratia*, and *Avicennia* are the plant species where *Vibrio* bacteria are most commonly found (Yahya et al. 2014; Yulma et al. 2017, 2020). The result of this study aligns with these findings, as *Vibrio* was most abundant in *S. alba*.

Identification of bacterial communities

This study established six amplicon libraries to represent the six mangrove species within the study area. After undergoing quality control and data processing, 173,270 amplicon sequence variants (ASVs) were identified, which could be attributed to 147 families and 218 genera based on the V3-V4 region of the 16S rRNA gene. Among these ASVs, the most prevalent genera were *Vibrio*, *Stenotrophomonas*, *Serratia*, *Pseudoalteromonas*, and *Achromobacter*. However, the abundance of these dominant bacterial genera exhibited significant variations among the different mangrove populations. Specifically, *Stenotrophomonas* and *Achromobacter* were more abundant in *Ceriops tagal*, while *Serratia* dominated in *R. apiculata*, and *Vibrio* was most abundant in *N. fruticans*, *S. alba*, and *R. mucronata*. Furthermore, *Pseudoalteromonas* exhibited higher abundance in the *R. stylosa* mangrove species (Figure 2).

The results reveal various microorganisms associated with each host mangrove species. It is worth emphasizing that microbial activities are pivotal in driving essential nutrient transformations within the mangrove ecosystem (Alongi 2014). Various microbial populations continuously convert nutrients from decomposed mangrove vegetation into forms, such as nitrogen, phosphorus, and other accessible nutrient sources utilized by mangrove trees (Kutty et al. 2021). These processes encompass methanogenesis, phosphate solubilization, sulfate reduction, and the synthesis of various compounds like antibiotics and enzymes (Wu and Lu 2015). Notably, genera like *Serratia* and *Stenotrophomonas* have been found to participate in the nitrogen cycle and carbon dioxide fixation (Dechavez et al. 2022; Meng et al. 2022). These nitrogen-fixing bacteria inhabit the rhizosphere and decompose leaves, pneumatophores, and mangrove sediments (Zhang et al. 2017a), where the denitrification process occurs, converting nitrates into nitrogen. This process is crucial for maintaining a dynamic nitrogen balance within mangroves (Wang et al. 2019) and acts as a vital sink for nitrogen, removing approximately 6% of nitrogen inputs into the environment (Wang et al. 2019; Zhang et al. 2020).

Noteworthy, microbes within the mangrove area also contribute significantly to global carbon cycling (Allard et al. 2020). Despite covering only a relatively small portion of the world's forests, approximately 3% of the total mangroves play a substantial role as carbon sinks, sequestering about 10% of the world's total carbon emissions (Jakovac et al. 2020). The sequestered carbon dioxide from the atmosphere is securely stored in anoxic sediments of mangroves, where it remains stable until disturbed (Chatting et al. 2022).

Apart from their role in nutrient cycling, there are phosphate-solubilizing microbes, such as certain *Vibrio* species (Thatoi et al. 2013), present in mangroves. Mangrove mud effectively absorbs nitrates and phosphates from tidal waters (Sari and Fitri 2019). Since most inorganic phosphate in the mangrove area is insoluble (Behera et al. 2016), phosphate-solubilizing bacteria are crucial in releasing soluble phosphate into the pore water. Phosphorus is an essential nutrient for plants, similar to nitrogen (Fatimah et al. 2023), making these phosphate-solubilizing microorganisms (PSMs) necessary for providing phosphorus in the mangrove environment and facilitating plant growth (Teymouri et al. 2016).

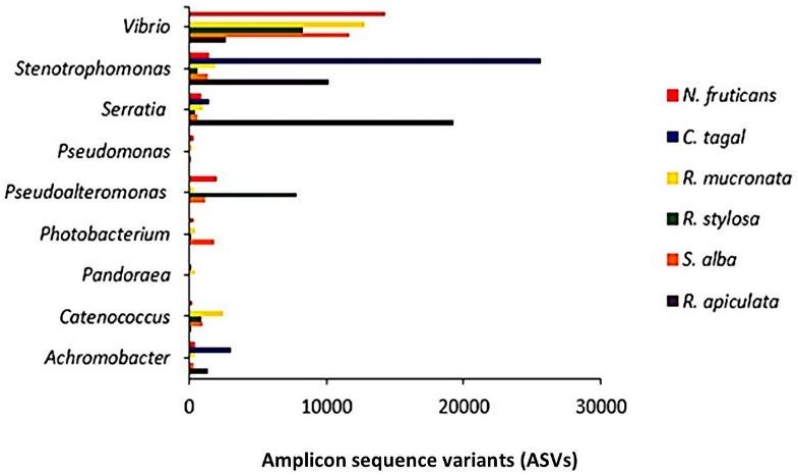

Figure 2. Amplicon sequence variants (ASVs) count comparison of bacterial groups at the genus level of the six mangrove host species

Microorganisms found in mangroves are not only beneficial to the plants themselves because some species, like those from the genus *Pseudoalteromonas*, exhibit pharmaceutically relevant activities that can be harnessed for medicinal purposes (Offret et al. 2016; Eze et al. 2023). According to Srinivasan et al. (2021), *Pseudoalteromonas* spp. possess diverse bioactive and therapeutic potentials, often involving metabolites. The synthesis of secondary metabolites by marine bacteria opens new avenues for discovering and developing unique natural compounds (Valliappan et al. 2014). Moreover, marine bacteria hold significant promise as a reservoir for creating innovative therapeutic substances (Karthikeyan et al. 2022). For instance, through thorough screening and rigorous examination, marine bacteria may provide us with antimicrobial agents essential for combating drug-resistant pathogens in the next 100 years (Ancheeva et al. 2018).

Alpha diversity

Mangroves host highly diverse microbial communities, some of which include species that are not closely related to known bacteria and have been found to inhabit and function within mangrove roots (Purahong et al. 2019). When assessing the alpha diversity of the bacterial community using the Shannon index, it was observed that *S. alba* displayed the highest diversity, with a value of 2.75 among all the mangrove species examined (Figure 3). Results were consistent with previous studies where *S. alba* consistently exhibits the highest diversity in microbial communities (Muwawa et al. 2021; Dechavez et al. 2022). The genus *Sonneratia* is often found in river estuaries and can thrive in environments characterized by high environmental stress and poor conditions (Sari et al. 2019). These mangroves can grow in sediments with a higher clay-to-silt ratio than sandy soils (Trangia 2022). They possess a unique system of aerial roots or structures that resemble snorkels emerging from the muddy substrate. These structures facilitate oxygen exchange in saturated soil (Hongwiset et al. 2022). The aerial roots of *Sonneratia* are particularly well-adapted to highly saline habitats, such as the seaward-most regions of the forest (Raganas and Magcale-Macandog 2020).

The density of underground fine roots gradually increases toward the central regions, these roots provide crucial support for the sediment that binds and stabilizes the mangrove forest (Karimi et al. 2022). These compartments within mangrove trees significantly shape the microbiome communities, primarily promoting the host's survival (Wainwright et al. 2023). The structure and canopy cover of *S. alba* contributes to plant productivity, carbon fixation, and the burial of organic matter, making it a rich carbon source for microbial communities and fostering bacterial diversity (Chen et al. 2016; Zhuang et al. 2020).

In addition, the observed zonation in mangrove forests results from a complex interplay of biotic and abiotic factors (Ahmed et al. 2023). Different zones within the mangrove forest offer distinct environmental conditions (Dangan-Galon et al. 2016). For instance, some areas may have higher salinity levels, while others are less saline due to freshwater input from nearby rivers. These varying conditions significantly impact the types of microbes that can thrive in each zone (Drew et al. 2021). These factors create unique environmental conditions in different zones, consequently influencing the structure and diversity of microbial communities (Hou et al. 2017). An in-depth understanding of these dynamics is essential for grasping the ecological significance of mangroves and their role within coastal ecosystems (Afonso et al. 2021).

Functional prediction through KEGG pathways

The prediction of the functional composition of the microbiome at the KEGG pathway level 2 was conducted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. This analysis unveiled functional predictions related to metabolism, xenobiotic biodegradation, and bioremediation for the bacterial communities in the six mangrove species examined in this study (Figure 4). Functional prediction provides an initial overview of the potential capabilities of bacterial communities thriving in the mangrove ecosystem (Muwawa et al. 2021).

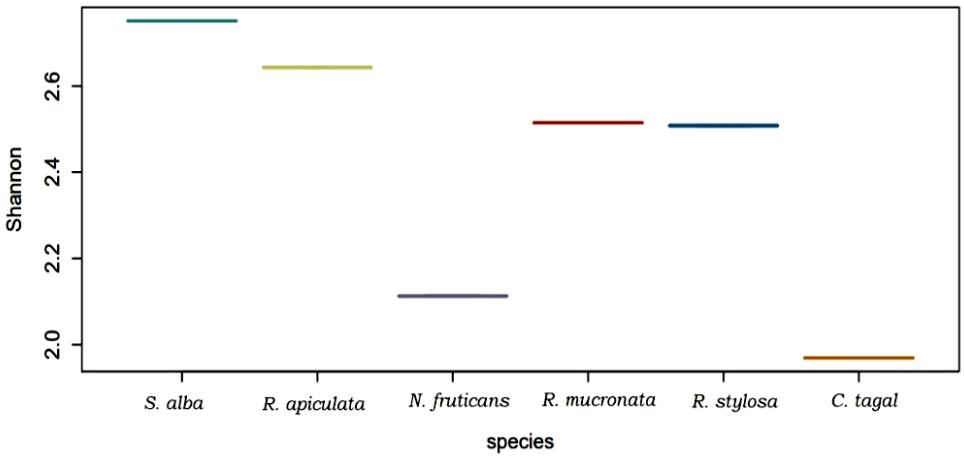

Figure 3. Shannon diversity index of bacterial communities from the six mangrove host species generated from the Parallel Meta-Suite (PMS) pipeline

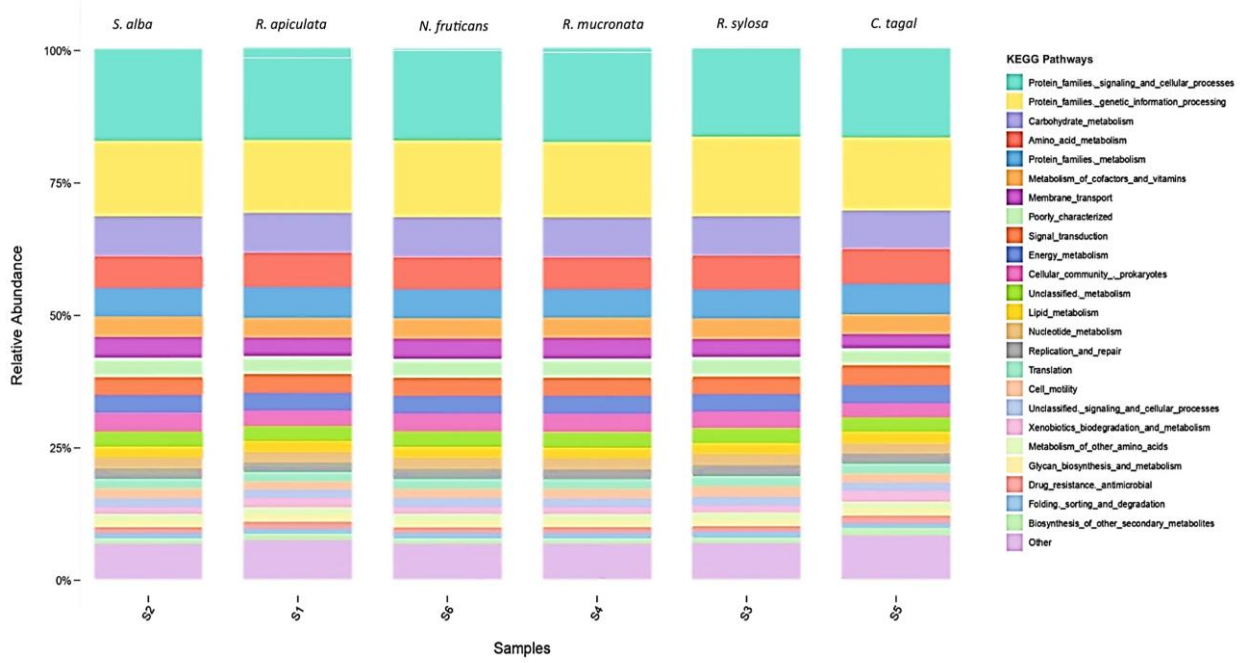

Figure 4. PICRUSt analysis of predictive functional analysis of bacterial communities as represented by KEGG pathways for the six species of mangroves in the study area.

Cultured bacteria derived from mangroves have already been studied for their bioremediation potential (Cabral et al. 2019). In this process, microorganisms can transform contaminants into less harmful substances, influencing biogeochemical cycles (Cabral et al. 2013; Dixit et al. 2015; Brar et al. 2017). Bacteria isolated from mangrove sediment have been shown to degrade persistent pollutants (Ren and Ni 2023); therefore, identifying and assessing microorganisms capable of degrading these pollutants is highly beneficial for bioremediation efforts and restoring natural ecosystems. This is particularly important for breaking down and transforming compounds like plastic polymers (Auta et al. 2017). Plastic pollution is widespread, especially in marine ecosystems such as mangroves (Liu et al. 2023). Globally, mangroves are recognized as one of the most heavily polluted areas because they accumulate various types of plastic from the ocean, including microplastics (Jambeck et al. 2015).

The accumulation of microplastics (MPs) in the environment has far-reaching negative consequences for organisms, soil, and water. In particular, the pervasive presence of microplastics (MPs) is a global concern that impacts aquatic ecosystems and threatens existing organisms (Sajjad et al. 2022; Yuan et al. 2022). For instance, within the mangrove ecosystem, microplastics can induce false satiety, pathological stress, reduced growth rates, and reproductive complications among organisms (Zhang et al. 2017b; Bour et al. 2018). Furthermore, microplastics can bind to other chemicals or metals, acting as carriers for toxic substances and creating synergistic effects (de Ruijter et al. 2020). On the other hand, microorganisms are inherently opportunistic and adaptable to various environments, enabling them to potentially transform a wide range of compounds, including plastic polymers (Auta et al. 2017). In polymer degradation, microorganisms initially adhere to the polymer's surface, initiating microbial colonization (Zeenat et al. 2021). Subsequently, these microorganisms release extracellular enzymes that attach to the polymer, leading to hydrolysis and its breakdown (Amobonye et al. 2021). As the polymer undergoes degradation, it forms lower-weight polymer fragments that eventually mineralize into carbon dioxide and water, serving as an energy source for microorganisms (Wang et al. 2017).

Previous research has shown that specific microorganisms can break down particular types of plastic particles. For example, polypropylene (PP) can be degraded by microorganisms such as *Rhizopus arrhizus* A.Fisch., *Vibrio*, and *Pseudomonas* sp. (Raghul et al. 2014; Ren and Ni 2023). *Bacillus* sp. can degrade polyethylene (PE), and bacteria like *Klebsiella*, *Citrobacter*, and a species of the genus *Pandoraea* can degrade polystyrene (PS) (Venkatesh et al. 2021; Cai et al. 2023; Ren and Ni 2023). These functional bacterial communities within ecosystems enhance the organisms' survival rates (Thatoi et al. 2013).

There are intriguing genera known for their involvement in xenobiotic biodegradation. For example, the genus *Serratia* demonstrates a broad range of metabolic abilities and can degrade organic compounds, including xenobiotics (Ma et al. 2015). Additionally, certain species of *Stenotrophomonas* and *Achromobacter* have been shown to degrade xenobiotic compounds, such as aromatic hydrocarbons and pesticides (Hong et al. 2017; Parte et al. 2017). The genus *Achromobacter* can also play a role in bioremediation of crude oil-polluted environments in

oceans, as it can produce biosurfactants that aid in the bioremediation process (Marzec-Grządziel and Gałązka 2023).

Certain species within the genus *Photobacterium* have demonstrated efficiency in phytoremediation to combat mercury contamination in soil (Mathew et al. 2015). Furthermore, the genus *Catenococcus*, a gram-negative and facultatively anaerobic group of bacteria from the Vibrionaceae family, contains only one known species, *Catenococcus thiocyclus*. While it has not been directly linked to xenobiotic degradation, it is found in marine volcanic areas and is associated with sulfur cycling. It can oxidize thiosulfate to tetrathionate, although the specific benefits of this reaction remain unknown to date, with limited scientific literature available on this genus (Sorokin et al. 1996). Similarly, the genus *Pseudoalteromonas* has been identified as involved in alginate degradation (Ito et al. 2019). Alginate, synthesized by brown algae and broken down by heterotrophic bacteria, represents a significant organic carbon source in marine ecosystems (Xu et al. 2021).

In conclusion, the most frequently observed amplicon sequence variants (ASVs) were associated with the genera *Vibrio*, *Stenotrophomonas*, *Serratia*, *Pseudoalteromonas*, and *Achromobacter*. Mangroves, particularly *Sonneratia alba*, exhibited the highest bacterial alpha diversity, signifying that the structure and coverage of these mangroves provide an ideal environment for the flourishing of microorganisms. The presence of microbes and their potential for bioremediation holds promise for conserving and protecting our natural environment. Our result contributes to the ongoing efforts to ecologically restore the once extensive and thriving mangrove forest at the research site.

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