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RESEARCH ARTICLE

Dynamics of Root-Associated Microbiomes in Ratooning Sugarcane: Insights from Shotgun Metagenomic

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Abstract Ratooning in sugarcane is crucial for agriculture sustainability, but the changes in microbial communities during consecutive ratooning remain unclear. We employed a shotgun metagenomic approach using Illumina 454 sequencing to investigate the root-associated microbial community in four sugarcane plantation sites, with three sites in the Dompu Regency and one in the Madiun Regency. The results revealed variations in the root-associated microbiome between ratoon and plant cane, as well as across different sites. pH, total organic carbon, and nitrogen were found to influence the community structure. Across the four sites, we identified 395 species, including 382 species of bacteria, 10 species of archaea, and 3 species of eukaryotes. In the ratoon plants of Dompu, the most abundant species was *Paraburkholderia caribensis* (40%), whereas in the plant cane dominated by *Rhizobium pusense* (16%)., In Madiun, the predominant species in ratoon plants was *Rhodanobacter sp.* DHG33 (20%) and *Paraburkholderia sp.* SOS3 (16%). These bacterial species may serve as key contributors to plant growth in ratooning plantations in these regions. The fungal community declined during the ratooning cycle but increased at later stages, possibly indicating a more stable fungal community in mature ratoon crops. The community structure varied, with bacteria more abundant in Dompu Regency's ratoon plants and archaea in Madiun Regency's ratoon plants. Understanding microbial dynamics across these four sites will support the development of sustainable strategies for ratooning practices in sugarcane production.

Keywords: Bioprospecting, continuous farming, microbial community, molecular ecology, sugarcane microbiome.

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Introduction

Sugarcane is an important industrial crop with strategic significance and diverse uses, including food (sugar), energy (bioethanol), and biomaterials [1,2]. The sugar production industry contributes about 80% of the world's total sugar, producing approximately 183 million metric tons in 2023-2024 [3–5]. . In Indonesia, the sugar industry is crucial for food security and economic growth. The country's average sugar production ranges from 2.1 to 2.3 million tons, while national demand is 6 million tons per year, necessitating imports [5–7]. Indonesia is the world's largest sugar importer and aims to achieve self-sufficiency in sugar for household consumption by 2028 and for industrial use by 2030. To meet these goals, Indonesia focuses on expanding sugarcane planting areas and improving productivity and sugar yield [6,7].

Effective cultivation management is essential for maintaining long-term productivity and soil fertility in sugarcane plantations [8]. Ratooning, a common practice in sugarcane management, involves growing new crops from the buds of previously harvested sugarcane stubble. The initial plant cane (PC) crop is

followed by the ratoon (RT) crop, which can be harvested multiple times, offering numerous advantages [9]. Ratooning reduces costs for planting materials and tillage, allows earlier maturation, and lowers production costs by eliminating the need for seedbed preparation, seed material, and planting operations, reducing fertilizer input, manure, and irrigation inputs [4,9,10]. It also extends the crushing schedule for sugar factories by contributing to early tissue dehydration and nitrogen removal [11].

Similar to other continuous cropping systems, ratooning can lead to soil issues, including reduced soil fertility and health, lower soil pH, and the development of soil-borne diseases. These issues can result in decreased crop yields, lower yield quality, stunted growth, and a negative impact on the long-term sustainability of agriculture [10,12]. It also reduces the activity of important soil enzymes like invertase, urease, and acid phosphatase, affecting soil fertility and nutrient availability [12,13]. These changes harm soil health and agricultural productivity and are linked to increased chemical use, higher cultivation costs, decreased agrobiodiversity, and increased greenhouse gas emissions, reducing growers' income [14].

The plant rhizosphere hosts distinct microorganisms such as bacteria, archaea, fungi, nematodes, protozoa, and invertebrates, which interact with one another and with their plant hosts in complex ways that influence plant health and productivity. These interactions are modulated by plant growth, environmental factors, and microbial community composition, forming a mutually beneficial relationship essential for sustainable agriculture [15–18]. In sugarcane plantations, particularly under ratooning systems, understanding and harnessing these microbial communities is crucial for enhancing crop performance and long-term soil health [12,17,19,20]. Shotgun metagenomic studies have shown considerable promise in identifying previously uncharacterized microbial taxa and predicting dominant metabolic pathways across diverse environments. This approach allows for in-depth profiling of soil- and plant-associated microbiomes, enabling the detection of beneficial microbes, pathogen surveillance, and optimization of nutrient cycling [21]. However, microbiome research specific to sugarcane ratooning systems in Indonesia's sugarcane plantation remains limited, underscoring the need for targeted studies. Moreover, the impact of farming practices impact on these microorganisms varies based on soil type, crop rotation, and climate [22], further emphasizing the importance of context-specific investigations to optimize microbial contributions to sustainable and productive sugarcane cultivation. The long-term effect of these practices on plant-microorganisms interactions may be underestimated. This study explored the microbial community structure in three locations of sugarcane plantations in Dompu Regency, a region with the potential for increased sugarcane productivity and expansion [23]. Additionally, we examined a long-established sugarcane plantation in Madiun Regency [24]. Investigating the composition and distribution of the root-associated microbiome in sugarcane plantations of these regions can offer a meaningful understanding of the ecological and agronomic factors influencing the development of these microbial communities.

Materials and Methods

Sampling Site Description

Rhizosphere soil and root samples were collected from sugarcane monoculture systems at different sites between July and August 2023. Dompu Regency: L2, ninth ratooning cycle, sampled eight months after cutting (GPS: S 08°16'51.95" E 117°48'25.08"); L3, third ratooning cycle, sampled one month after cutting (GPS: S 08°18'33.97" E 117°47'25.55"). L6, a first-year crop from seed cane, was sampled five months after planting (GPS: S 08°19'50.66" E 117°48'08.78"). Madiun Regency: LN1, fourth ratooning cycle, sampled eight months after cutting (GPS: S 06°50'44.57" E 108°37'01.67").

Dompu Regency has a drought climate with temperature ranges from 22°C to 31°C and 1,400 mm of rainfall in 2023. Madiun Regency has temperatures between 29°C and 34°C and 1,829 mm of rainfall in 2023. All sites used chemical fertilizer (ZA 600-700 kg/ha, NPK 500-600 kg/ha, KCl 100-150 kg/ha), rain feeding, and mechanized land tilling systems

Sample Collection

Five healthy clump were sampled at each site to keep the sampling practical while still capturing microbial diversity. The clumps were spread out to reflect local variation. The number of samples is in line with previous studies using metagenomics to identify main microbial patterns. I. For each clump, 3 individual crop roots were taken from young, moderate, and oldest stalks. An area of approximately 0.5 to 1 m^2 and 0.5 to 0.8 m deep was excavated around the plant clumps to extract the entire plant. Stalks and leaves were cut and separated from the roots, and extensive soil aggregates were removed by shaking the roots. The root pieces were then placed in a sterile plastic container and kept in a cool box before transfer to the lab. Rhizosphere soil samples from each site were pooled and composited before being analyzed. Soil analysis followed the Indonesian National Standard (SNI) for soil analysis.

Root Sample Preparation

Root samples from each replicate within the same site were combined. About 100 g of pooled root pieces were shaken in 500 mL of sterile ice-cold phosphate-buffered saline (PBS) at 150 rpm for 30 min. The solution was filtered and centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was centrifuged again, and the pellet containing rhizosphere microbes was frozen in liquid nitrogen and stored at −80 °C. The PBS-tween20-washed roots were rewashed in 250 to 500 mL PBS_tween20 (7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.0, and 0.05% tween20 and shaken at 150 rpm for 15 min. This was repeated three times, followed by a final rinse with ice-cold PBS and the removal of excess water with sterile tissue. About 100 g of washed roots were carved and blended in ice-cold PBS. The mixture was filtered and centrifuged at 200×g for 5 min at 4°C to remove debris, then centrifuged again at 6,000 rpm for 15 min at 4 °C. The resulting pellet contained an endophytic microbe [25]. These rhizosphere and root sample pellets were used for DNA extraction.

DNA Extraction and Shotgun Sequencing

The rhizosphere and root endosphere sample pellets from the previous step were extracted following the DNA extraction protocol of the Qiagen DNeasy Power Soil Kit (USA), following the manufacturer's protocol with 250 mg of pellets, each sample was processed in triplicate Total genomic DNA was assessed both qualitatively and quantitatively using gel electrophoresis and a NanoDrop spectrophotometer. The DNA samples were then sent for sequencing to PT. Genetica Science Shotgun Metagenomic Service (NexGen Sequencing Service, 1st base, Singapore).

Quality Control and Removal of Host DNA

Initially, sequence reads totalling 29 Gb across eight samples were subjected to quality assessment using FastQC v0.12.1, with subsequent consolidation of results using MultiQC v1.18 [26]. Following quality checks, adapter sequences were trimmed, and reads with a Phred score below 15 were discarded using Trimmomatic v0.39 [27]. The trimming parameters included settings as follows: PE, ILLUMINACLIP: adapter.fa:2:30:10:3:true, LEADING:2, TRAILING:2, SLIDING WINDOW 4:15, and MINLEN: 36. Notably, host DNA contamination was identified and removed by aligning reads against the sugarcane genome (*Saccharum officinarum*, GenBank accession number: GCA_020631735.1) using Bbduk (BbMap v39.01).

Taxonomic Profiling and Diversity Analysis

We used MetaPhlAn v4.1.0 [28], leveraging marker genes around 1 million microbial genomes (mpa_vJun23_CHOCOPhlAnSGB_202307). BowTie2 v2.5.3 [29] aligned reads to the MetaPhlAn marker gene database for species identification and quantification. The analysis was conducted using the R program v4.3.1 (R Core Team, 2023). Species abundance was visualized with ComplexUpset v1.3.6. The Principal Component Analysis (PCA) and Redundancy Analysis (RDA) were performed to explore relationships between soil characteristics and microbial community structure. Alpha diversity, including Richness, Shannon, and Simpson indices, was calculated using a custom R script, while beta diversity (Bray-Curtis dissimilarity) was visualized with Principal Coordinates Analysis (PCoA) using an R package vegan v2.6.6.1. Metagenomic analysis was extended with MetaWRAP v1.3.2 [30], using metaSPAdes [31] for assembly and Kraken2 v2.0.9-beta for taxonomic profiling [32] against KRAKEN2 PlusPF (k2_pluspfp_20240112), NCBI_nt, and NCBI_tax databases

Results and Discussion

Root-Associated Microbial Abundance

The Illumina platform generated 454 M raw sequence reads from 8 shotgun metagenome libraries with paired-end sequencing, ranging from 23.3 M to 36.0 M reads per sample. The mean sequence length of paired-end reads was 150 bp, with an average Phred score above 30. After quality filtering, which included host decontamination and base quality trimming, approximately 7.79% of reads were discarded. The remaining 418.6 M high-quality sequences were used for subsequent analyses. Results from Kraken2 v2.0.9-beta analysis in Figure 1 showed that bacteria were the most common microbes in the sugarcane roots environment, constituting 86% to 99% of the reads. Among these, *Proteobacteria* is the most abundant phylum, followed by other bacterial phyla such as *Actinobacteria* (11.97%) and *Bacteroidota* (5.41%). This result is consistent with other studies showing plant roots preferentially take up these phyla [33–35]. They can be attributed to their metabolic versatility, plant growth-promoting capabilities, and most importantly, their remarkable adaptability to changing environmental conditions [33–35]. This adaptability, along with beneficial interaction with plants, enables them to thrive in the nutrient-rich, dynamic environment created by ratooning practice, ultimately supporting plant health and productivity.

The study showed the relatively low presence of archaea (less than 3%) and eukaryotes (less than 1%) suggests that these microbial groups are less prominent in the sugarcane roots, although certain archaea (*Thaumarchaeota*) and fungi (*Ascomycota*) were detected in L6 site (0.29% in root endosphere and 0.77% in rhizosphere) where newly planted sugarcane was grown, but were not found in the ratooning sites. Additionally, the presence of specific microbial groups like *Chloroflexi*, *Verrucomicrobia*, and *Planctomycetota* in distinct samples (e.g., LN1) indicates that microbial communities can vary spatially within the sugarcane rhizosphere and root endosphere. A study by Ren *et al*. [36] on root rot disease found that the sugarcane fungal community was mainly dominated by *Ascomycota* and *Basidiomycota*, with some unclassified groups. *Ascomycota* was more abundant in the rhizosphere soil of susceptible sugarcane varieties than in moderate or resistant ones.

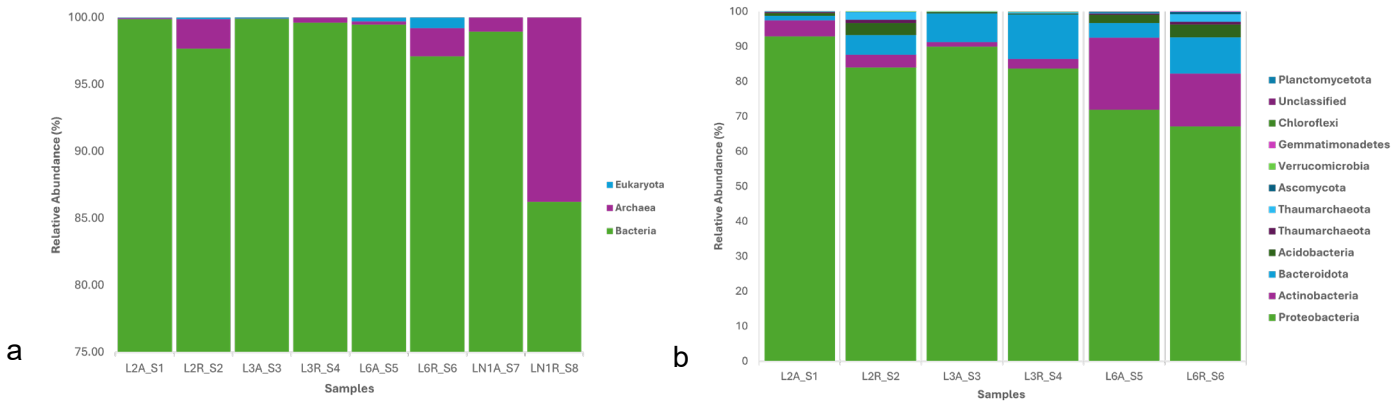

Figure 1. Relative abundance microbes in Dompu Regency: L2 site root endosphere (L2A_S1) and rhizosphere (L2R_S2), L3 site root endosphere (L3A_S3) and rhizosphere (L3R_S4), L6 site root endosphere (L6A_S5) and rhizosphere (L6R_S6), and Madiun Regency: LN1 site root endosphere (LN1A_S7) and rhizosphere (LN1R_S8) at: (a) Kingdom level and (b) Phyla level

Root-Associated Microbial Diversity

The richness indices of L6A_S5 were the highest, with 196 different species observed, followed by L6R_S6, with 139 different species observed. Interestingly, despite L2R_S2 having more species, the evenness of microbial abundances in sample LN1A_S7 resulted in higher diversity (Figure. 2). The root-associated microbiome is crucial in supporting plant growth, health, productivity, and resilience against various stresses [37–41]. The composition of the root-associated microbiome mainly comes from soil microbes that migrate to the roots, and their diversity generally decreases from bulk soil to rhizosphere soil to rhizoplane to endosphere [37,42,43]. In contrast, we observed that the microbial community was generally more abundant in the root endosphere than in the rhizosphere for all sites, except for the L2 site. Thisresult can be attributed to both methodological factors, such as root leaching efficiency, sampling techniques, PCR amplification, and sequencing methods [44,45], and biological factors, including host-driven selection and the specialized environment of the endosphere [43,46,47]. On the other hand, this result agrees with Beckers *et al*.[48], who found that the diversity of rhizosphere microbiomes in field-grown poplar is lower than that of endosphere microbiomes. Their study also showed that the microbiomes in stems and leaves are more specialized, with each plant part providing a unique environment for bacterial communities. In the sugarcane ratooning practice, roots select and house microbes internally during the first planting and retain them during the ratooning cycle. In addition, the endosphere is a more limited environment than the rhizosphere, with fewer resources and distinct physical and chemical conditions. These factors create selective pressure that shapes unique microbial communities with specialized functions [48–50].

Our diversity analyses showed that the ratooning cycle and sampling sites influenced root-associated microbiota's taxonomic and functional profiles. Newly planted sugarcane (L6) was more diverse (Shannon index) than the 3rd, 4th and 9th ratoon sugarcane (Figure 2). Monoculture practices and ratooning can decrease the diversity and activity of soil and root-associated microbes, reducing beneficial microbes and increasing harmful ones [51,52]. However, [53] found no significant differences in bacterial richness and community diversity in the rhizosphere soil of three-year ratooning sugarcane compared to newly planted sugarcane. It could imply that under certain conditions, ratooning may not result in the anticipated negative impacts on microbial diversity, possibly due to adaptive shifts in microbial communities over time or the specific environmental conditions of the study site.

Furthermore, the PCoA analysis using the Bray-Curtis algorithm revealed significant differences in the microbial communities across the sites. PC1 accounted for 40.21% of the total variability, while PC2 explained 31.28% (Figure 3). Samples from Dompu Regency were separated from those of Madiun Regency. Within the Dompu samples, L6 formed a distinct cluster separate from L2 and L3 (Figure 3). Notably, L6 consisted of newly planted sugarcane, while L2 and L3 were from ratooning cycles plants. This indicates that microbial composition differs both between regions and between newly planted and ratooning sugarcane plants.

A total 395 of different species were observed among the samples indicating a relatively high level of microbial diversity in the study areas. Among those species identified, 110 different species were observed in L2A_S1; 127 were observed in L2R_S2, 90 were observed in L3A_S3, 89 were observed in L3R_S4, 196 were observed in L6A_S5, 139 were observed in L6R_S6, 83 were observed in LN1A_S7, and LN1R_S8 had the lowest different species (59). The most unique species was presented in L6A_S1, which accounts for 51 unique species. Highlighting that the microbial community in newly planted sugarcane is more specialized or distinct compared to those of ratooning.

Fifteen unique species were present in all samples of Dompu Regency (L2, L3, L6). This suggests that there are core species common to this region, potentially reflecting shared environmental conditions. Interestingly, no common species were found between samples from Dompu and Madiun sites. The absence of common species between Dompu and Madiun suggests a clear regional differentiation in microbial communities. Dompu and Madiun regions differ significantly in soil pH and texture (Table 1), which may contribute to the absence of shared microbial species between the two regions. This finding is consistent with the study by Xia *et al.***[54]**, which identified soil pH and texture as key factors influencing the composition of soil microbial communities. This indicates that, despite having the same ratooning practice, the microbial populations in the two sites are most influenced by different soil factors,and potentially also by variations in climate andmanagement practices, leading to distinct microbial profiles. Thirteen species were present in L6 and L2, eleventh species were present in L6 and L3. The root endosphere of the L3 site was observed as the lowest unique species, and only three different species were identified (Figure 4).

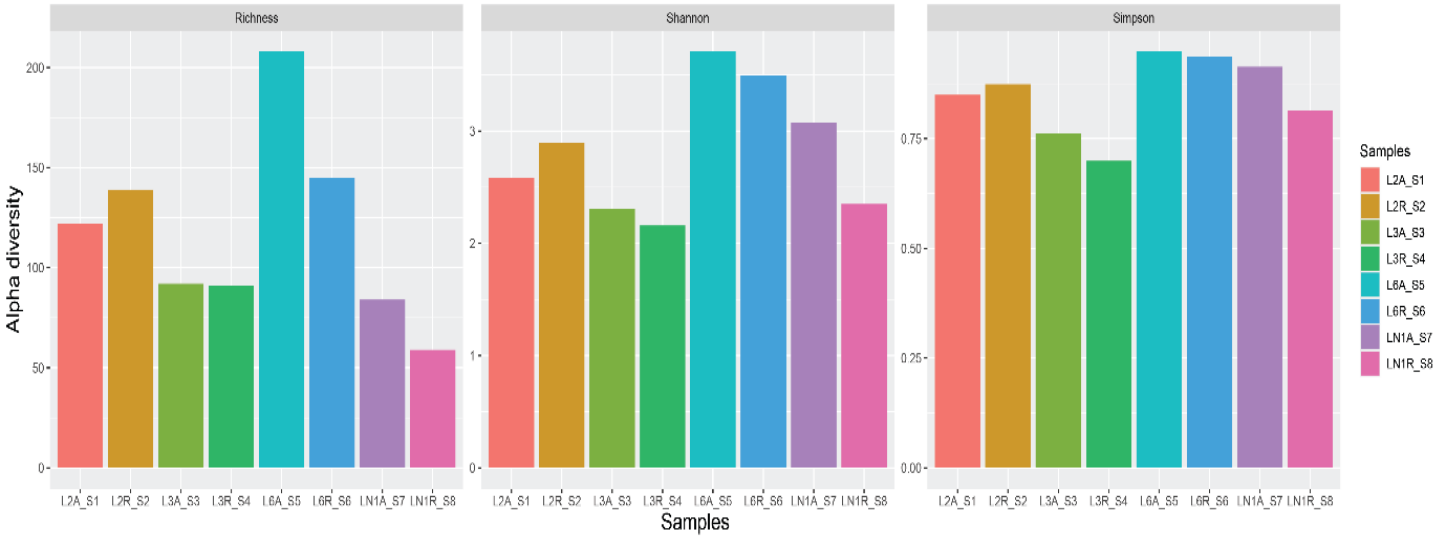

Figure 2. Observed species (richness), Shannon and Simpson's index representing richness and evenness at the species level

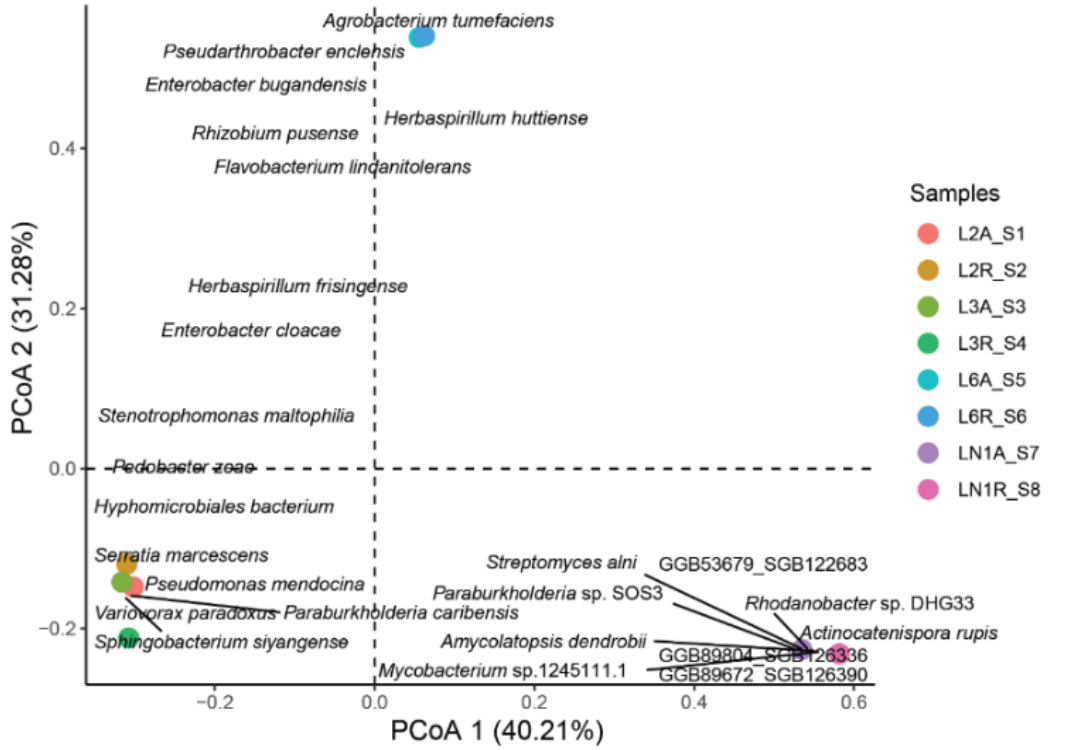

Figure 3. The PCoA graph of root-associate microbiomes in sugarcane plantation sites

The Richness of Root-Associated Microbial Community

In this study, root-associated bacterial community richness exhibited distinct patterns across different sites, with varying species composition and abundance. At the L2 site, *Paraburkholderia caribensis* was the most abundant species, accounting for 32.44% in L2A_S1 and 28.4% in L2R1_S2. Other prominent species in L2A_S1 included *Hyphomicrobiales* bacterium (24.64%), *Serratia marcescens* (8.8%), *Kosakonia radicincitans* (4.99%), and *Enterobacter cloacae* (4.19%). In L2R_S2, the dominant species were *Serratia marcescens* (17.60%), *Hyphomicrobiales* bacterium (8.51%), *Variovorax paradoxus* (6.77%), and *Rhizobium mesoamericanum* (4.48%). At the L3 site, *Paraburkholderia caribensis* was also the most abundant species, representing 45.61% in the root endosphere and 53.11% in the rhizosphere. In L3A_S3, other dominant species included *Variovorax paradoxus* (13.73%), *Hyphomicrobiales* bacterium (5.61%), *Sphingobacterium siyangense* (4.37%), and *Pseudomonas mendocina* (3.29%). In L3R_S4, the key species were *Variovorax paradoxus* (10.42%), *Flavobacterium anhuiense* (5.01%), *Pedobacter zeae* (4.47%), and *Herbaspirillum frisingense* (2.70%). At the L6 site, the top species in the root endosphere were *Rhizobium pusense* (16.20%), *Pseudarthrobacter enclensis* (7.08%), *Enterobacter bugandensis* (6.86%), *Agrobacterium tumefaciens* (5.62%), and *Hyphomicrobiales* bacterium (5.55%). In the rhizosphere, the dominant species were *Rhizobium pusense* (16.03%), *Enterobacter bugandensis* (13.72%), *Flavobacterium lindanitolerans* (6.61%), *Pseudarthrobacter enclensis* (6.42%), and *Agrobacterium tumefaciens* (4.99%). The bacteria found to be most abundant at each site, *Paraburkholderia caribensis*, *Hyphomicrobiales,Kosakonia radicincitans*, *Variovorax paradoxus, Rhizobium mesoamericanum, Rhizobium pusense, Flavobacterium anhuiense* have all been identified in numerous studies as plant growth-promoting bacteria [55–58], with established roles in supporting sugarcane growth [59]. In contrast, *Agrobacterium tumefaciens*, although detected, is known to cause crown gall disease in a wide range of plants species [60]. Additionally, *Enterobacter cloacae* was reported by Macedo-Raygoza *et al.* [61] as a potential endophyte of banana plants, functioning both as an antagonist against the fungal pathogen *Pseudocercospora fijiensis*, which causes Black Sigatoka disease in bananas, and promoting plant growth in nutrient-deficient soils through nitrogen transfer. This may also have beneficial effects on sugarcane growth and health.At the LN1 site, the root endosphere was dominated by *Rhodanobacter sp. DHG33* (20.11%), followed by *Paraburkholderia sp. SOS3* (15.61%), *Streptomyces alni* (7.13%), *Sphingomonas oligoaromativorans* (6.46%), and *Actinocatenispora rupis* (5.93%). In the rhizosphere, archaea were more prevalent, with *Mycobacterium sp. 1245111_1* (7.83%), *Rhodanobacter sp. DHG33* (4.65%), *Nitrososphaera sp. AFS* (3.81%), *Amycolatopsis dendrobii* (1.95%), and *Trebonia kvetii* (1.38%). Both of the most abundant

species , *Rhodanobacter sp* in the endosphere and *Mycobacterium sp.* in the rgizosphere have been reported as plant growth-promoting that exhibiting multiple beneficial traits [62,63], and is well adapted to low pH environments [64].

Proteobacteria were the dominant phylum across all sites, representing over 70% of bacterial diversity, except for at LN1, where Firmicutes and Thaumarchaeota were more prominent. The next most common phyla at all sites were Actinobacteria and Bacteroidota, consistent with studies showing that plant roots preferentially uptake these phyla due to their metabolic versatility, plant growth-promoting abilities, and adaptability to changing environments [33–35].This adaptability, combined with beneficial interactions with plants, enables these taxa to thrive in the nutrient-rich and dynamic environment created by ratooning practices, supporting plant health and productivity. In contrast, at LN1, Firmicutes were more abundant in the rhizosphere, with some evidence suggesting their role in disease suppression [65], while Thaumarchaeota, more abundant in the root endosphere, plays a significant role in soil nitrification, aiding to nitrogen cycling and carbon dioxide fixation [66]. These findings suggest a high level of bacterial dominance and variability in the microbial composition depending on the environmental conditions of different sampling sites.

PCA analysis indicated that the samples from Dompu (L2, L3, L6) clustered separately from those from Madiun (LN1). Moreover, newly planted sugarcane samples from L6 were distinct from ratooning samples (L2 and L3), further suggesting that site and planting type influence the bacterial community composition. These findings highlight the complex interactions within microbial communities and emphasize the importance of both abundant and less abundant taxa in maintaining soil health and supporting plant growth by maintaining microbial networks [67].

Fungi observed in this study were exclusively from the phylum *Ascomycota*, which is known for its crucial role in carbon and nitrogen cycling, contributing to soil stability, decaying plant biomass, and endophytic communications with plants in arid ecosystems [68]. Among the *Ascomycota* fungi identified, *Candida intermedia* was found at low abundance in L3A_S3 (0.038%), L6A_S5 (0.159%), and L6R_S6 (0.77%). *Candida blattae* was detected in L2A_S1 (0.029%) and L2R_S2 (0.1%), while *Penicillium sp. occtitanis* appeared in L2A_S1 (0.01%) and L6A_S5 (0.13%).

Ascomycota was notably abundant in newly planted sugarcane (L6) in both the rhizosphere and endosphere, but it was absent in the rhizosphere of third ratoon plants (L3) and both the rhizosphere and endosphere of fourth ratoon plants (LN1). Interestingly, *Ascomycota* was present again in the ninth ratoon plants (L2). This pattern suggests that fungal communities were more prominent in newly planted sugarcane, with a decrease in abundance during the ratooning cycles, possibly due to nutrient depletion, increased microbial competition, soil compaction, and pathogen accumulation [46,69]. However, by the ninth ratoon cycle, the microbial community likely adapted, with the accumulation of organic matter and shifts in community dynamics leading to a resurgence of fungal abundance. *Proteobacteria* and *Ascomycota* have been reported in many studies to play a crucial role in disease-suppressive soils and in maintaining soil nutrient status [47,70,71].

Growing evidence indicates that rhizosphere microbes are crucial for reducing nutrient stress and protecting against pathogens by using root exudates to attract specific beneficial bacteria and fungi [47]. Fungi strongly influence the root microbiome and are more affected by host characteristics compared to bacteria, although both are influenced by the soil environment and the host plant [72]. In our study, fungal abundance decreased with increasing ratoon cycles, reflecting changes in soil nutrients and microbial interactions. The decline in the fungal community in third and fourth ratooning cycle may negatively impact plant health and yield due to several factors, including increased susceptibility to soil-borne diseases, reduced nutrient availability, and altered plant defense mechanisms [73,74]. Wang *et al*. [75] reported that available potassium and phosphorus in continuous cropping systems were key soil factors influencing shifts in fungal community structure. This may help explain the reduced fungal abundance observed in both the rhizosphere and endosphere of ratoon crops compared to the plant cane, as ratooning can lead to nutrient depletion in the soil. The resurgence of fungi in the ninth ratoon may indicate a shift toward a more stable microbial community after several cycles of plant growth and microbial adaptation. These findings underscore the dynamic nature of microbial communities in sugarcane ecosystems and highlight the importance of both fungal and bacterial populations in supporting plant health across different growth stages.

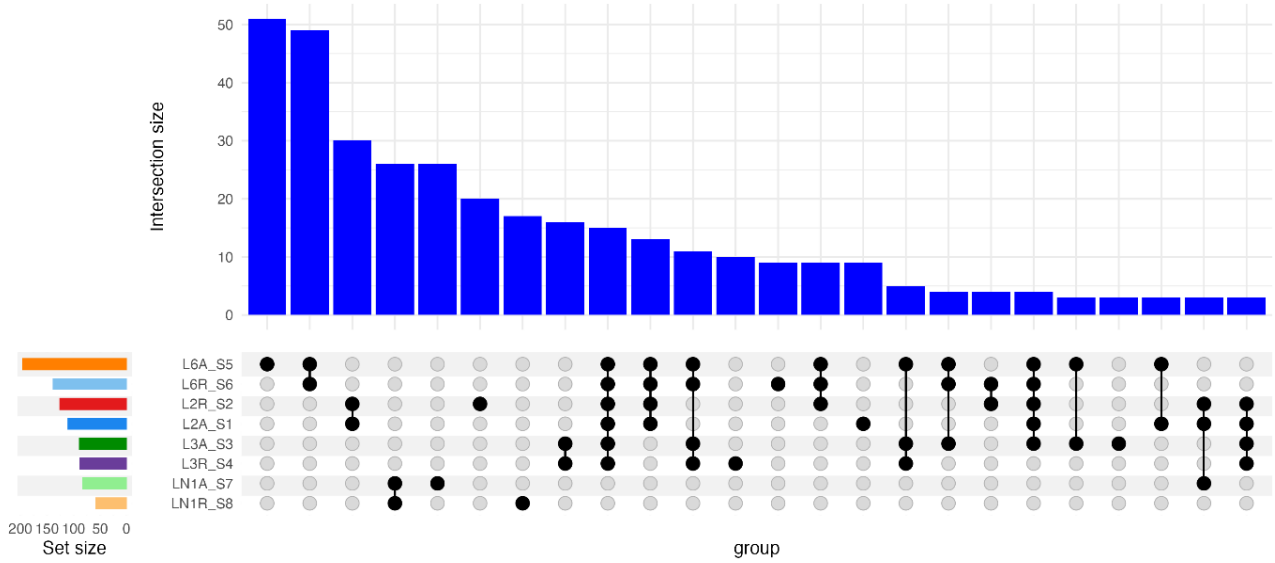

Figure 4. Venn diagram using ComplexUpset v1.3.6 of species abundances among samples

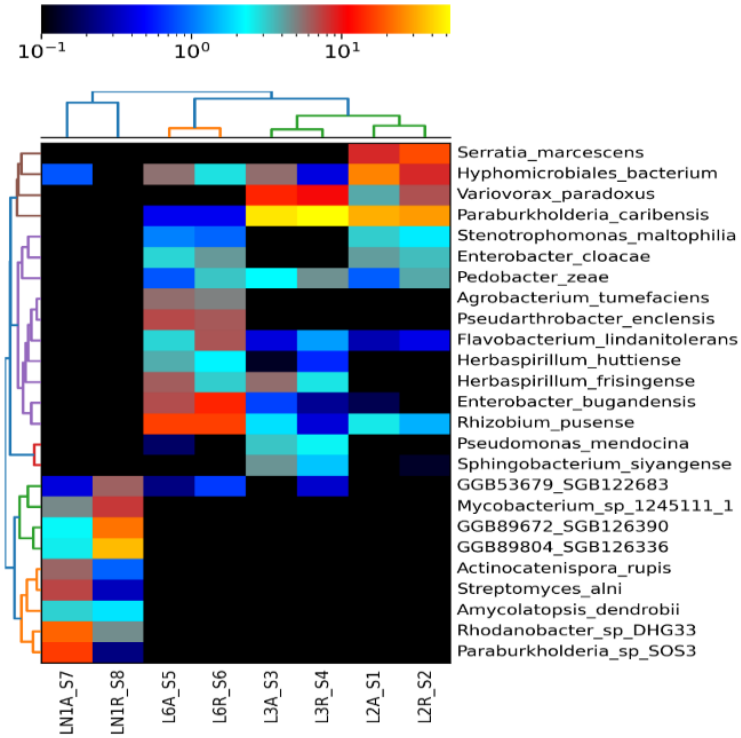

Figure 5. Heatmap of the species abundance across samples

Archaea were most abundant in LN1, making up 9.95% of the rhizosphere and 0.39% of the root endosphere. No archaea were found in L2 samples. In L3, archaea were 0.35% in the rhizosphere and 0.05% in the root endosphere, while in L6, archaea made up 0.68% of the rhizosphere and 0.22% of the root endosphere. These results show that archaea are most common in the rhizosphere of LN1, with lower amounts in the root endosphere. The absence of archaea in L2 suggests that this site does not support archaea as much as others. Archaea were found in small amounts in L3 and L6, with higher levels in the rhizosphere. According to Taffler *et al.* [76], Archaea may interact with plants by promoting

growth through auxin biosynthesis, supplying nutrients, and protecting against stress. Their ability to interact with fungi and their role in the carbon and nitrogen cycles, including CO_2 and N_2 fixation, further highlight their importance in plant health and nutrient cycling. In addition, Liu *et al*. [77] pointed out that Archaeal communities have an important role in sustaining microbial stability, which can reduce soil sickness and boost crop yields in continuous soybean farming.

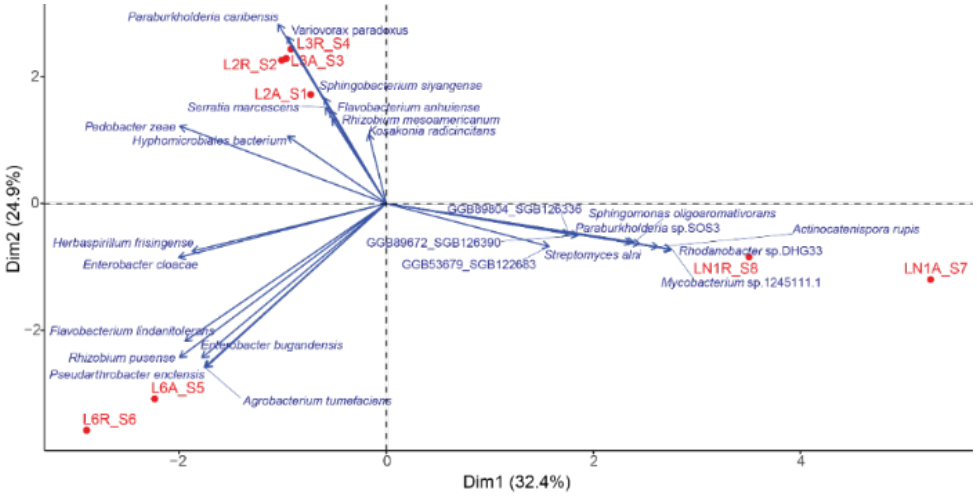

Figure 6. The principal component analysis graph of species abundance of root-associated sugarcane's microbiome

Table 1. Soil physicochemical properties of sampling

Attributes	Sampling Site			
	L2	L3	L6	LN1
pH	6.7	6.8	7.2	4.8
Soil Organic Carbon (%)	1.81	0.82	2.9	1.21
Nitrogen-Total (%)	0.14	0.09	0.22	0.15
C/N Ratio	13	9	13	8
Available P2O5 (mg/kg)	44.91	23.31	15.51	71.07
Potential P2O5 (mg/100 g)	429.4	259.96	300.75	69.38
Potential K2O(mg/100 g)	189.6	288.83	245.85	29.99
Cation Exchanfe Capacity (cmol(+)/kg)	7.44	6.02	10.39	12.45
Al3+ Exchangeable (cmol(+)/kg)	< 0.04	< 0.04	< 0.04	0.76
H+ Exchangeable (cmol(+)/kg)	0.12	0.08	0.13	0.17
K+ (cmol(+)/kg)	0.6	1.04	1.12	0.33
Na+ (cmol(+)/kg)	0.07	0.14	0.07	0.11
Ca2+ (cmol(+)/kg)	5.98	3.26	12.12	7.02
Mg2+ (cmol(+)/kg)	1.5	0.77	2.1	1.05
Base saturation %)	100	87	100	68
Sand (%)	97	89	94	31
Silt (%)	2	10	5	48
Clay (%)	1	1	1	21

Effects of Soil Physicochemical Properties

Soil physicochemical properties for each site are provided in Table 1. Redundancy analysis (RDA) showed that axes 1 and 2 explained 54.97% and 90.84% of the variability in the rhizosphere (Figure 7a) and 58.44% and 93.30% in the root endosphere (Figure 7b). Key factors like soil pH, total organic carbon (C), and total nitrogen content (N) were linked to the presence of *Enterobacter bugandensis*, *Rhizobium pusense*, and *Hyphomicrobiales bacterium* in both the rhizosphere and root endosphere. Additionally,

Flavobacterium lindanitolerans was specific to the rhizosphere, while *Pseudarthrobacter enclensis* was specific to the root endosphere. Furthermore, *Paraburkholderia caribensis*, *Variovorax paradoxus*, and *Serratia marcescens* showed a reverse correlation with nitrogen content but were strongly connected to *GGB89804 SGB126336*, *GGB89672 SGB126390*, and *Mycobacterium sp.*

The first two axes of PCA explained 79.2% of variation based on the top five species (Figure 8a) and 63.7% based on the top phyla (Figure 8b). The PCA analysis identified three distinct clusters: L2 and L3 grouped together, while L6 and LN1 separated. Notably, LN1 samples exhibited the greatest dispersion at the phyla level, with clear separation between the root endosphere and rhizosphere. Further analysis revealed that *Verrucomicrobia* was associated with cation exchange capacity (CEC), *Proteobacteria* with the carbon-nitrogen (CN) ratio, and *Bacteroidota* with potassium potential.

Our findings suggest that soil pH, organic carbon, and total nitrogen are key factors shaping the microbiome of sugarcane roots. According to previous research, soil pH is a critical factor affecting bacterial diversity, with the highest diversity typically found in neutral soils [54]. Another important factor is soil texture, with silt-based soils supporting greater microbial biodiversity and biomass than sandy soils. Smaller soil particles offer more surfaces for bacterial growth, which aids in the decomposition of organic matter and nutrient cycling, particularly nitrogen [78]. These factors significantly influence the structure and composition of the root-associated microbiome in sugarcane. The PCA analysis further underscores the importance of soil characteristics, such as CEC, CN ratio, and potassium potential, in shaping microbial communities across different sites.

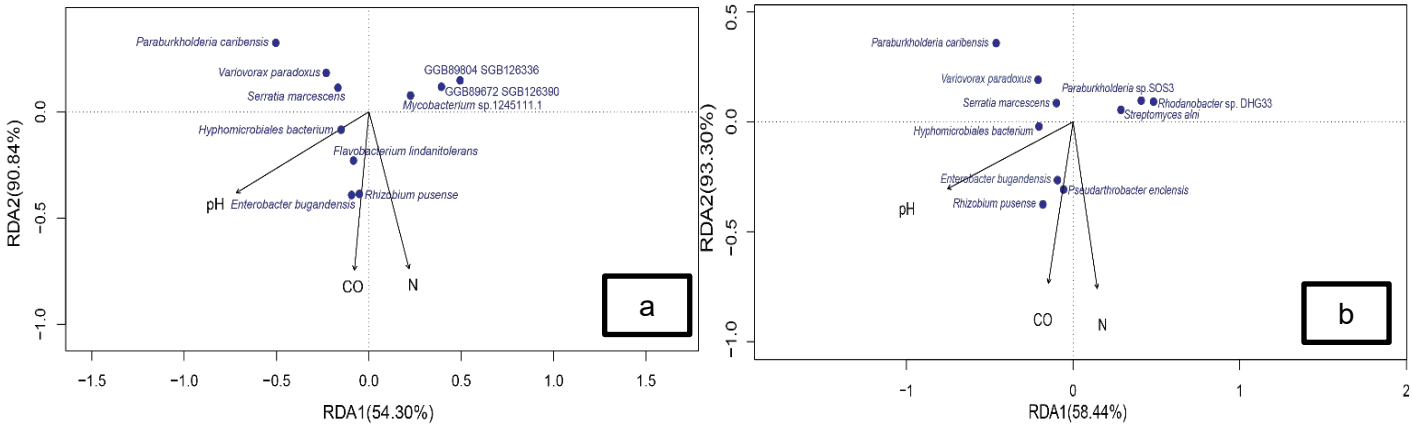

Figure 7. Redundancy analysis of microbial community and soil properties factors in (a) rhizosphere; (b) root endosphere.

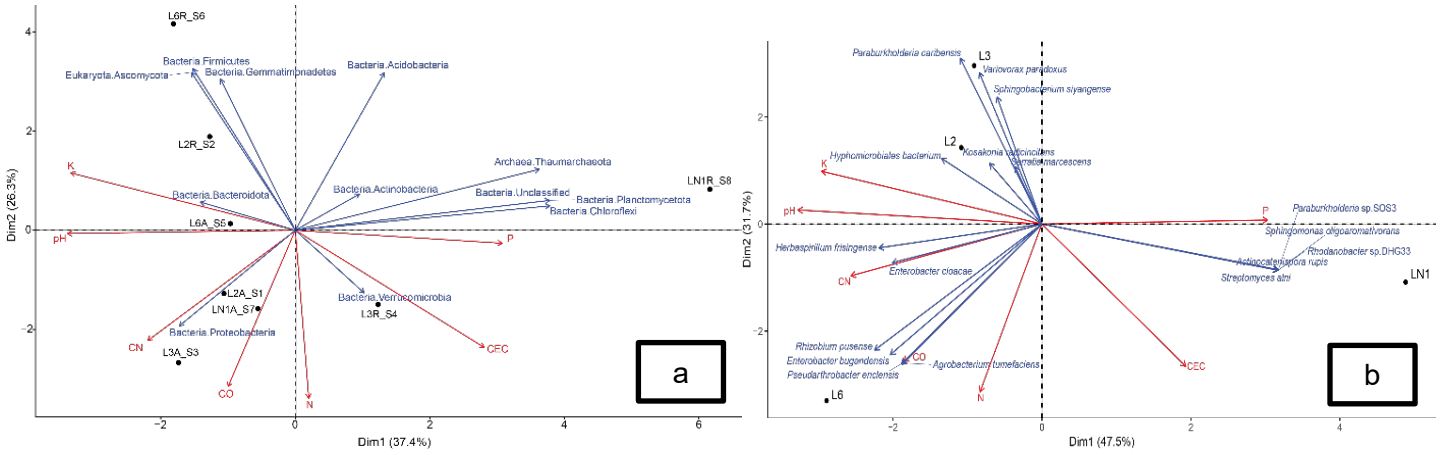

Figure 8. Principal component analysis of the microbial community and soil properties at phylum level (a) and species level (b) in each sample site

Conclusions

In conclusion, this study highlights the dynamic changes in the root-associated microbiome of sugarcane across different ratooning cycles and geographic locations. Our findings reveal that bacterial communities, particularly those dominated by *Rhizobium pusense*, *Paraburkholderia caribensis*, and *Rhodanobacter sp. DHG33* may play a significant role in shaping soil health and promoting plant growth, especially on ratoon plants. Soil properties such as pH, organic carbon, and nitrogen content were identified as key factors influencing microbial diversity, with bacterial richness generally surpassing fungal richness. However, intensive and continuous sugarcane farming without crop rotation may lead to the accumulation of harmful microbes, ultimately reducing soil fertility and disrupting the activity of essential soil enzymes. These results emphasize the importance of managing soil health and microbial communities to sustain productive and environmentally responsible sugarcane production. Further research could explore the functional roles of specific microbial taxa in nutrient cycling and disease suppression would provide valuable insights into developing more resilient and sustainable farming practices.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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