



16S Metagenomic Analysis of Microbial Diversity from the Rhizosphere of Lentil (*Lens culinaris* Medik) Collected from Tezpur, Assam

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10.18805/LR-5570

ABSTRACT

Background: Lentil plants enhance soil quality and productivity by forming symbiotic relationships with a diverse group of microbes present in the rhizosphere. The Cultivar-specific interactions, Rhizobium strains and plant genetics influence microbial diversity in the rhizosphere. This study aims to evaluate and compare bacterial diversity in lentil rhizospheres using metagenomics and 16S rRNA gene sequencing.

Methods: Rhizosphere soil samples from lentil cultivated sites were used for sequencing analysis. Genomic DNA was isolated from the rhizospheric soil using a standard protocol. Sanger/Illumina sequencing with a read length of 1.9 was performed on the extracted genomic DNA. Kraken 2 was used for taxonomic classification. Alpha diversity was measured in metagenomic samples to assess diversity.

Result: The present research study identifies Proteobacteria as the dominant phylum and the important taxon *Rhizobium leguminosarum* in the lentil rhizosphere microbiome. At the class level, *Gammaproteobacteria* were the most dominant (64.23%), followed by *Alphaproteobacteria* (4.633%), *Betaproteobacteria* (2.042%), *Deltaproteobacteria* (1.691%), *Epsilonproteobacteria* (0.2565%), *Actinobacteria* (5.376%) and *Rubrobacteria* (0.2303%). Further studies will focus on metabolic pathways, nutrient cycle, stress tolerance and pathogen suppression. These microbial communities could improve crop quality and sustainability in a fluctuating climate.

Key words: 16S rRNA, Gene sequencing, Kraken 2, Lentil (*Lens culinaris* Medik), Metagenomics, Microbial diversity.

INTRODUCTION

Lentil (*Lens culinaris* Medik. subsp. *culinaris*) is an important cool-season legume that contributes significantly to household nutritional security in developing countries. This protein-rich crop is an excellent choice for rice-fallow areas, helping to meet the protein needs of a vegetarian society. Lentil plants form a symbiotic relationship with various microbial populations, which enhances soil health and boosts crop production through biological nitrogen fixation (BNF) (Müller *et al.*, 2016). The microbiome composition of dynamic rhizospheres alters soil functions and enhances crop growth by stimulating beneficial plant-microbe interactions (Baudoin *et al.*, 2003; Philippot *et al.*, 2013; Bakker *et al.*, 2015; Schlaeppi *et al.*, 2015; Lu *et al.*, 2018; Feng *et al.*, 2019; Liu *et al.*, 2019; Marques *et al.*, 2019). Moreover, different agroforestry systems and seasonal variations significantly affect soil chemical properties and nutrient availability (Singroha *et al.*, 2025).

Roots of plants interact with various microorganisms, including bacteria, fungi, viruses, algae and archaea (Tringe *et al.*, 2005; Badri and Vivanco, 2009). The relationship between plants and microorganisms promotes growth, facilitates nutrient flow and enhances resistance to pathogens and stress. In return, plants provide roughly 15% of their nitrogen and close to 20% of their fixed carbon in the form of assimilates to support the microbiomes (Lopes *et al.*, 2016; Dong *et al.*, 2018; Wang *et al.*, 2019). Lentil crops form symbioses with various

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How to cite this article: Bordoloi, R., Das, A., Pegu, R.K. (2025). 16S Metagenomic Analysis of Microbial Diversity from the Rhizosphere of Lentil (*Lens culinaris* Medik) Collected from Tezpur, Assam. *Legume Research*. 1-9. doi: 10.18805/LR-5570.

Submitted: 15-09-2025 **Accepted:** 19-11-2025 **Online:** 05-12-2025

microorganisms in the rhizosphere, which helps improve both productivity and soil health. The diversity of rhizosphere microbiomes is determined by the physical and chemical factors of the rhizosphere, which, in turn, are partly influenced by the genetics of the host plant (Peiffer *et al.*, 2013). In the case of biological nitrogen fixation in lentils, the plant is also influenced by the host genotype, the strain of *Rhizobium* involved and cultivar-specific relationships (Zehr and Turner, 2001; Abi-Ghanem *et al.*, 2011; Müller *et al.*, 2016). Previous studies have shown that higher nitrogen fertilizer doses reduce the number of effective root nodules in soybean. Additionally, nitrogen application increases plant height, leaf number, root-to-shoot ratio, biomass, pod number and seed weight (Budiastuti *et al.*, 2025).

Metagenomics enables us to access functional genes, large genomic regions and even whole bacterial genomes that could not previously be cultivated (Rondon *et al.*, 2000; Gillespie *et al.*, 2002). In this study, we characterized and compared the bacterial diversity of the lentil rhizosphere using metagenomic techniques, 16S rRNA gene sequencing and next-generation sequencing (NGS).

The main objective of this study is to analyze microbial dynamics in the rhizosphere of root-nodulating lentils through metagenomic approaches and shotgun metagenome sequencing.

MATERIALS AND METHODS

Sample collection and study period

Rhizosphere soil samples were collected from lentil cultivation sites in Tezpur, Sonitpur district, Assam, around the mid-blooming stage. Using a brush, rhizosphere soil was collected from lentil roots. The sample was immediately snap-frozen in sterile test tubes and stored at -20°C (Gleridou *et al.*, 2023). The subsequent experimental works were conducted in the Plant Physiology and Biochemistry Laboratory of the Postgraduate Department of Botany at Darrang College, Tezpur, from October 2023 to December 2024. The metagenomic analysis of microbial diversity was conducted at the AGT Biosciences Laboratory in the Guwahati Biotech Park, Assam.

Rhizosphere soil samples from lentil cultivated sites were used for sequencing analysis. Genomic DNA was isolated from the rhizospheric soil using the methodology described by Lamontagne *et al.* (2002). A paired-end library with an insert size of approximately 466 bp was prepared for the sample following the guidelines provided by New England Biolabs, Inc. (Beverly, MA, USA). The sequencing library construction and template preparation processes were carried out, followed by Sanger/Illumina sequencing with a read length of 1.9, using the extracted genomic DNA.

To determine the structure and composition of the core bacterial communities in the lentil rhizosphere, the Illumina MiSeq sequencing platform was used for paired-end sequencing of DNA fragments. The V3-V4 region of the 16S rRNA gene was amplified using universal primers. The forward primer 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 1492R 5'-CGGTACCTTGTACGACTT-3' were used. The raw sequencing reads went through an initial screening using the Sanger quality score system (Phred+33). The analysis of the sequencing data and the quality assessment were performed in parallel using FastQC and MultiQC. These tools assessed several key metrics, including base quality scores, nucleotide composition, overall GC content at both the base level and within the sequence, sequence length distribution, N content, duplication rates, overrepresented sequences and K-mer content.

To maintain the quality of the data used for additional analyses, the raw reads were filtered. This process involved the following steps: (1) removing reads with adapter sequences, (2) excluding reads with ambiguous nucleotides greater than 10% (N > 10%) and (3) removing reads

where more than 50% of the bases had a quality score lower than 30. fastp, a verified preprocessor dedicated to FASTQ files, was utilized for the filtering and quality control processes. A separate workflow was created for quality control, where poor-quality reads were trimmed and adapter contaminants were removed.

Taxonomy analysis

Kraken 2 was used for taxonomic classification.

Taxonomy assignment 3596 (2.59%)

Taxonomic assignment involves using a hierarchical classification system to categorize organisms based on shared traits, lineage and ancestry. This system consists of eight main classifications: kingdom, phylum, class, order, family, genus and species.

Alpha diversity in metagenomics

Alpha diversity was measured in metagenomic samples to assess diversity. This was measured through the Observed Frequency, Chao1, ACE, Shannon, Simpson and Fisher indexes. Together, the indices exhibit a community's species richness (the number of species) and evenness (the distribution of species).

Phylogenetics plays a crucial role in metagenomic science by tracing the evolutionary pathways of different microbial populations within metagenomic datasets using phylogenetic trees. This helps determine taxonomic units, assess hierarchy, explore functional diversity and explain the functional capabilities of the community.

RESULTS AND DISCUSSION

Metagenomic datasets

Microbial communities in the rhizosphere directly impact the productivity and resilience of an agricultural ecosystem. These synergistic rhizobial activities are essential for supporting plant health and maintaining balance within natural ecosystems. Hence, in this study, we investigated the bacterial diversity and community structure in the metagenome of lentil (*Lens culinaris* Medik) rhizosphere soil using Illumina MiSeq sequencing.

To determine the structure and composition of the core bacterial communities in the lentil rhizosphere, we amplified the V3-V4 region of the 16S rRNA gene. From this, a total of 138,726 reads of high quality were generated. From these reads, 3,596 were classified as unclassified tags, while 135,130 were classified as tags (associated with taxonomic classification) (Table 3). The taxonomic analysis revealed that the 135130 classified reads were assigned to 23 taxonomic ranks at the kingdom level (Table 4).

FastQC (version 0.11.9) software indicates that the metagenomic reads have a 55% GC content (Fig 1). Following quality control (QC) and filtering of host sequences, a total of 138,726 high-quality reads were generated (Table 1 and Table 2). From these reads, 3,596 were classified as unclassified tags, while 135,130 were classified as tags (associated with taxonomic classification) (Table 3). The

taxonomic analysis further revealed that the 135,130 classified reads were assigned to 23 taxonomic ranks at the kingdom level, 43 at the phylum level, 79 at the class level, 122 at the order level, 197 at the family level, 275 at the genus level and to the species level (Table 4).

Abundance of soil microbial community

As to the abundance of the phyla, the most abundant was Proteobacteria (72.65%), followed by Firmicutes (8.395%) and the least abundant of the top 16 phyla was Synergistetes (0.5012%) (Heatmap 1). Firmicutes makes up slightly more than 8 per cent of the total reads and is the second most abundant phylum. Members of this phylum encompass a diverse range of metabolic types, including nitrogen-fixing

bacteria, sulfur-oxidizing bacteria and pathogens. The relative frequency of the phylum level is shown in Fig 2.

At the class level, *Gammaproteobacteria* were the most dominant (64.23%), followed by *Alphaproteobacteria* (4.633%), *Betaproteobacteria* (2.042%), *Deltaproteobacteria* (1.691%), *Epsilonproteobacteria* (0.2565%), *Actinobacteria* (5.376%) and *Rubrobacteria* (0.2303%) (Heatmap 2). The relative frequency of the class level is shown in Fig 3.

At the order level, the bacterial community is dominated by Enterobacterales (44.73%), followed by Vibrionales (7.62%), Pseudomonadales (3.39%) and Rhizobiales, which account for approximately 2%. At the family level, Enterobacteriaceae is the dominant family, making up 36.26% of all readings (Heatmap 3). Similar studies

Table 1: QC of raw reads.

Sample ID	Total sequence	Sequence with poor quality	Sequence length	GC %
BKMTG3003-GangaN1DR116s-R1	138.72800K	0	301	54
BKMTG3003-GangaN1DR116s-R2	138.72800K	0	301	55

Table 2: Trimming results.

Sample ID	Total reads before trimming	Total reads after trimming	Q20 bases before trimming	Q20 bases after trimming	Q30 bases before trimming	Q30 bases after trimming	Reads passed filters
BKMTG3003-GangaN1DR116s	138.728K	138.726K	36.37M (87.19%)	36.36M (87.19%)	33.31M (79.87%)	33.31M (79.87%)	138.72K

Table 3: Taxonomy analysis.

Sample ID	QC filtered reads	Number of reads	
		Classified	Unclassified
BKMTG3003-GangaN1DR116s	138726	135130 (97.41%)	3596 (2.59%)

Table 4: Taxonomy assignment table.

Sample ID	Phylum	Class	Order	Family	Genus	Species
BKMTG3003-GangaN1DR116s	23	43	79	122	197	275

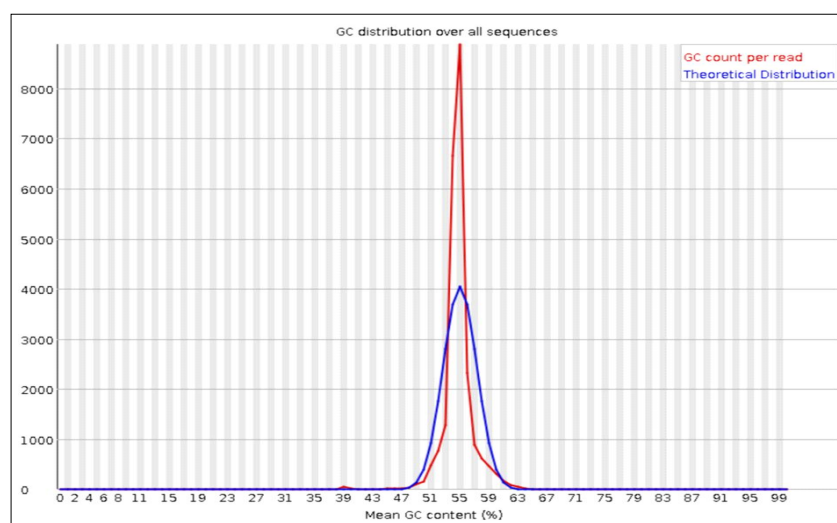


Fig 1: GC distribution of overall sequences.

indicate that the lentil rhizosphere is home to diverse microbes, including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and the genus *Rhodococcus*, which belongs to the phylum Actinobacteria (Martínez-Hidalgo and Hirsch, 2017; Pramanik *et al.*, 2000). The relative frequency of the order level is shown in Fig 4.

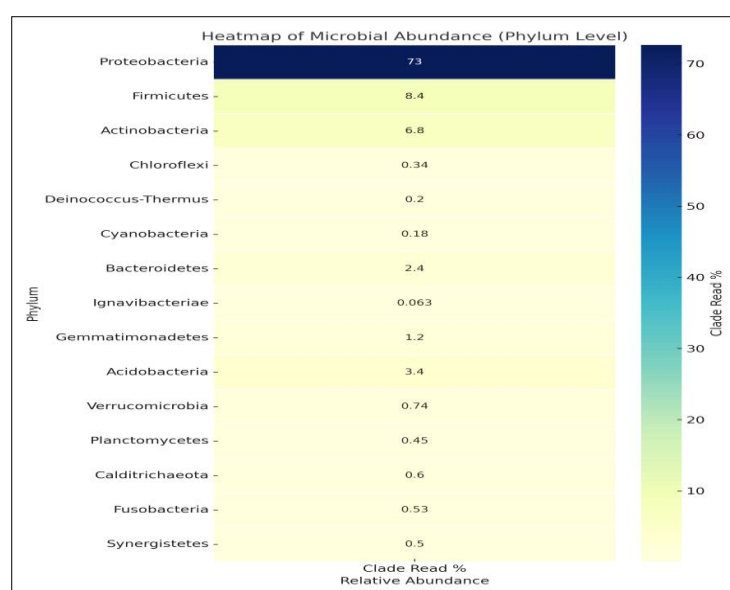
Among the top 15 contributing families, some, such as Halomonadaceae and Erwiniaceae, contribute very little to the total, suggesting a diversified but low-abundance background community (Heatmap 4). The relative frequency of families is shown in Fig 5.

Escherichia (17.92%) and *Salmonella* (9.26%) are the most abundant genera at the genus level, indicating that together, they account for over 27% of all reads. Typically, these are Enterobacteriaceae. An example of the many

genera in the bottom half is *Rhizobium*, *Kosakonia* and *Arsenophonus*, which collectively reach a relative abundance of only 0.2% (Heatmap 5).

The most represented species at the species level are *Salmonella enterica* (9.04%) and *Escherichia coli* (17.54%), which together account for over 26% of the total population (Heatmap 6). *Klebsiella pneumoniae*, with 3.38% of the readings, is also well-represented, which suggests it may have some significance to the community. *Rhizobium leguminosarum* is known for its symbiotic and nitrogen-fixing roles with plants, although it appears at relatively low relative abundances.

The alpha diversity metrics of the sample BKMTG3003-GangaN1DR116s demonstrate high species richness, as evidenced by the Chao1, ACE and Fisher indices and high



Heatmap 1: Relative abundance of the top 15 microbial phyla.

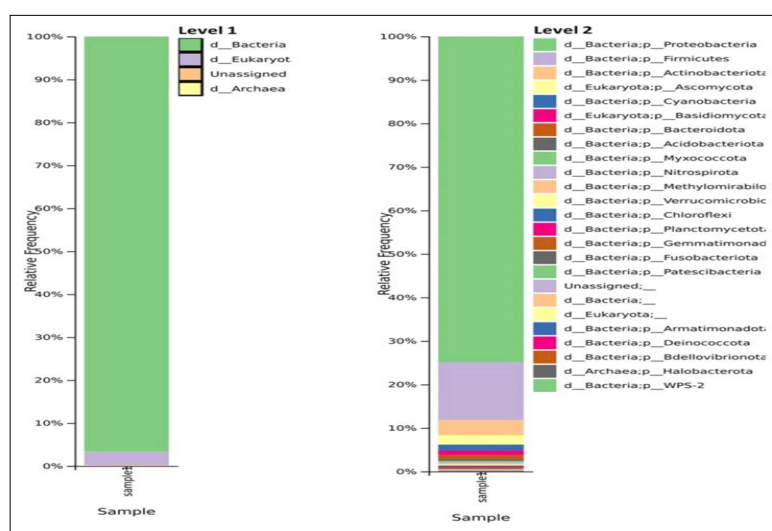
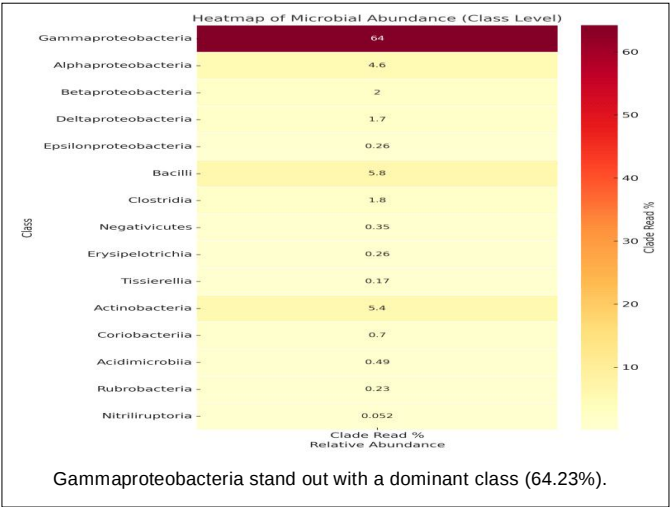
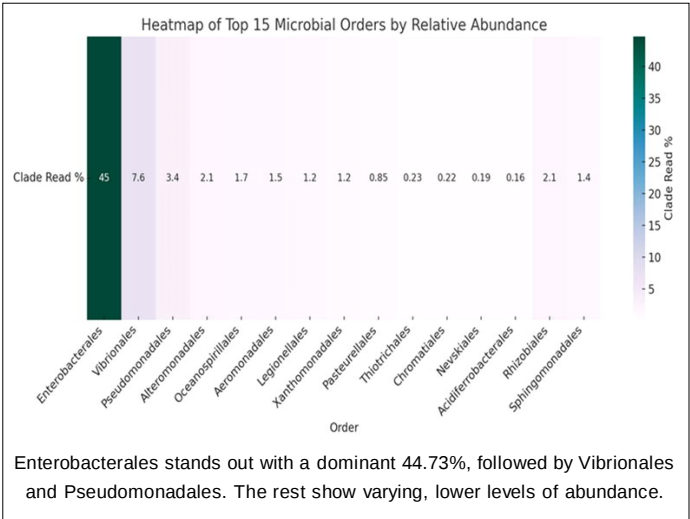


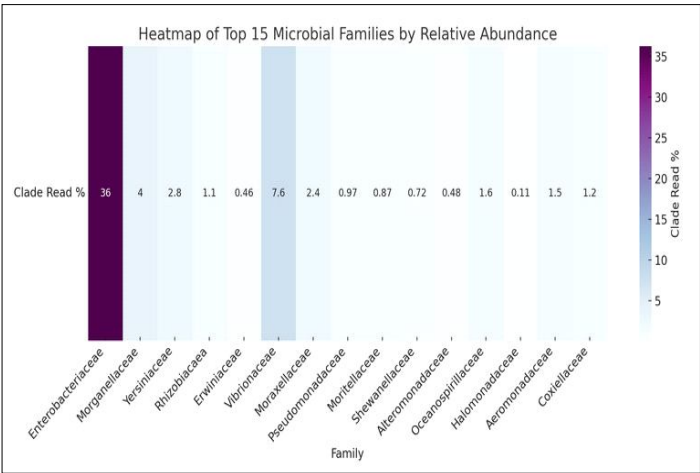
Fig 2: Relative frequency of microbial abundance at (a) the Domain level and (b) the Phylum level of individual samples after taxonomic classification.



Heatmap 2: Relative abundance of the top 15 microbial classes.



Heatmap 3: Relative abundance of the top 15 microbial orders.



Heatmap 4: Shows the relative abundance of the top 15 microbial families. As seen, Enterobacteriaceae is by far the most dominant, followed by Vibrionaceae and Morganellaceae, while others appear in lower but still relevant proportions.

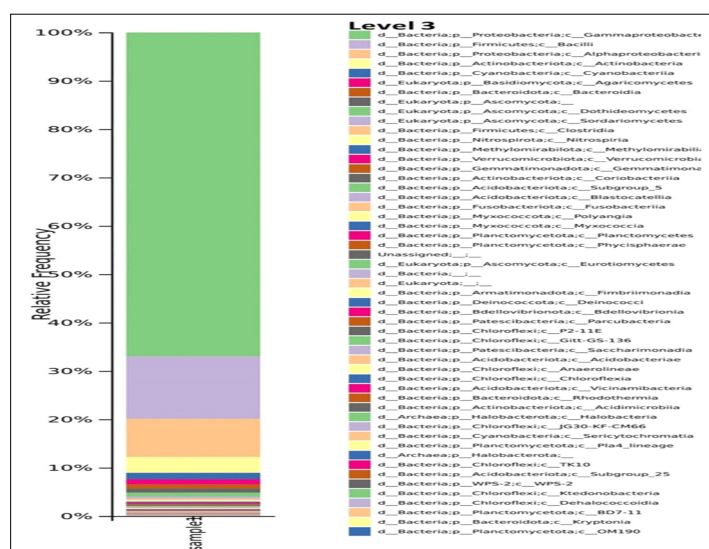
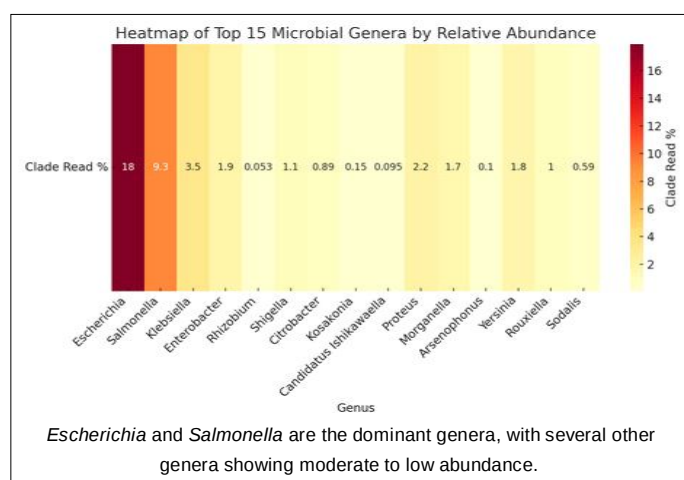
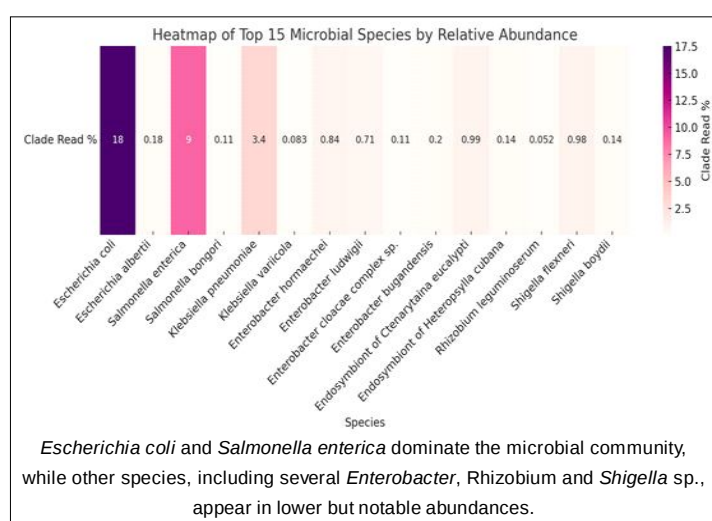


Fig 3: Relative frequency of microbial abundance at the Class level of individual samples after taxonomic classification.



Heatmap 5: Relative abundance of the top 15 microbial genera.



Heatmap 6: Relative abundance of the top 15 microbial species.

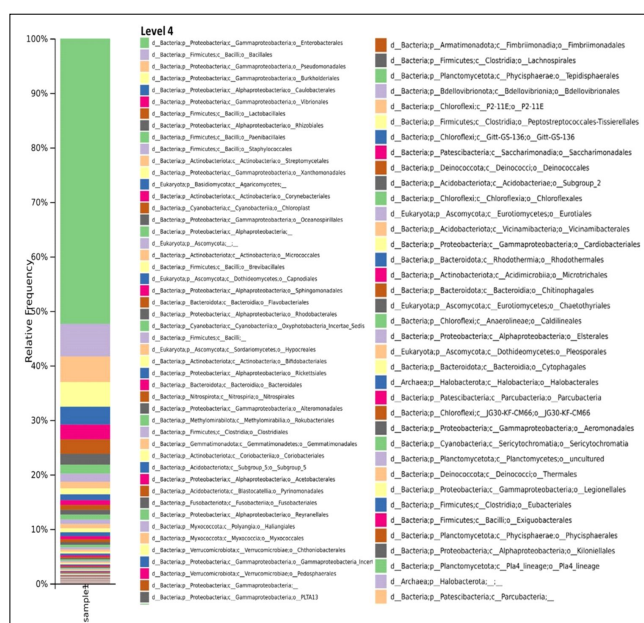


Fig 4: Relative frequency of microbial abundance at the Order level of individual samples after taxonomic classification.

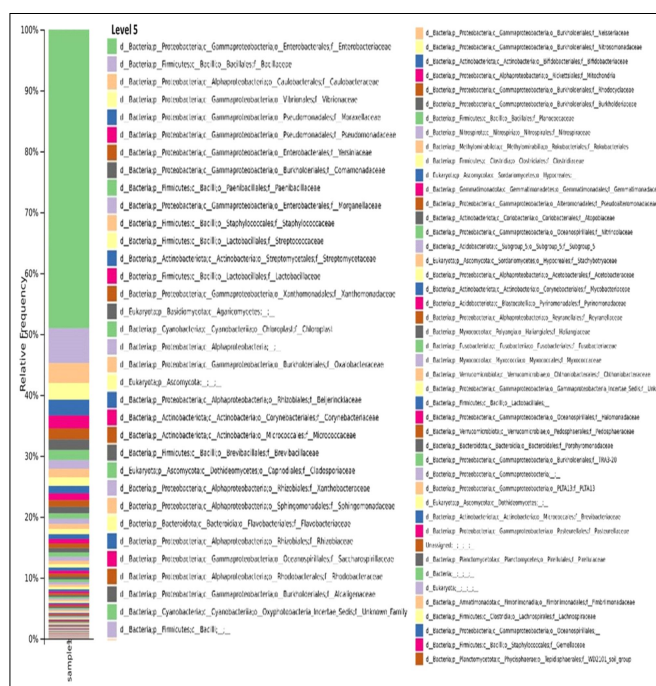


Fig 5: Relative frequency of microbial abundance at the Family level of individual samples after taxonomic classification.

Table 5: Alpha diversity.

Sample ID	Chao 1	ACE	Shannon	Simpson	Fisher
BKMTG3003-GangaN1DR116s	323.12	326.48	6.06	0.96	50.99

diversity and evenness, as shown by the Shannon and Simpson indices (Table 5).

In other studies, the rhizosphere of the lentil cultivars was found to be dominated by the bacterial phyla Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria

and Acidobacteria (Pramanik *et al.*, 2020). Satyanandam *et al.* (2022) suggested that the diversity of bacteria capable of nodulating the legume blackgram is high, including *Rhizobium*, *Bradyrhizobium* and *Achromobacter*. Shotgun metagenomics also revealed *Bradyrhizobium* and *Rhizobium*

as the dominant *Pseudomonadota* genera, both known for their symbiotic roles in promoting plant growth and fixing nitrogen (Lugtenberg and Kamilova, 2009).

CONCLUSION

The present research study shows taxonomic characterization of the lentil rhizosphere microbiome, revealing the dominance of the phylum Proteobacteria and functionally important taxa such as '*Rhizobium leguminosarum*'. Future functional metagenomic and metatranscriptomic studies are necessary to elucidate metabolic pathways related to the nutrient cycle, stress tolerance and pathogen suppression. However, it may also be possible to isolate and characterize beneficial strains and to develop targeted biofertilizers or microbial communities tailored to local agro-climatic conditions. Additional comparisons within lentil cultivars, across different soil types and farm practices, in combination with longitudinal monitoring, would also provide valuable insights into the interaction of genotype, microbiome and environment. These integrated strategies are promising for microbiome-mediated crop improvement, sustainable soil management and lentil management to maintain productivity in a fluctuating climate.

ACKNOWLEDGEMENT

We are grateful to Dr Akon Das for helping us with the metagenomic study of the lentil rhizosphere and we are also thankful to Dr. Bidhan Borah, Head, PG Department of Botany, Darrang College, Tezpur, for creating a friendly environment to carry out this study.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Ethical issues

None.

Conflict of interest

The authors declare that they have no conflicts of interest.

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