

# Metagenomic analysis of the rhizospheric soil of *Piper longum* L. established in D.E.I, Agra, India

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## Research Article

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# Abstract

The rhizosphere of medicinal plants contains a variety of microbial populations that influence plant health, nutrient uptake, and the synthesis of bioactive compounds. In this study, we used a culture-independent metagenomic approach to characterize the rhizospheric microbiota of *Piper longum*, a plant with significant medicinal potential in traditional medicine. High-throughput sequencing using the Illumina technology yielded 19.94 million paired-end reads (~ 5.92 Gb), which were assembled into 97,432 scaffolds, resulting in a genomic length of 52.26 Mb. In all, 45,876 genes were predicted and functionally identified across multiple databases. The phylum Proteobacteria is dominant in terms of taxonomic classification, with Nitrososphaera and *Candidatus Nitrososphaera gargensis* being the most common genus and species, respectively. A metabolically diverse and ecologically significant microbial population was indicated by functional profiling, which also showed enrichment in genes linked to metabolism, amino acid transport, and environmental adaptability. This work highlights the value of using microbial diversity for sustainable agriculture and enhanced phytochemical output while providing basic insights into the *P. longum* rhizosphere microbiome.

## 1. Introduction

One of the nature's most dynamic environments is the rhizosphere, which is the microecological interface between plant roots and soil. It is created by complex interactions between plants, soil, and a broad range of microorganisms, including bacteria, fungi, protozoa, and archaea (Philippot et al. 2013; Vetterlein et al. 2020). These microbial communities play a crucial role in plant health by affecting growth regulation, nutrient uptake, stress tolerance, and disease defense. Consequently, the rhizospheric microbiota is preferentially shaped by the root exudates that plants release, which are made up of sugars, amino acids, organic acids, and secondary metabolites (Ali and Glick 2024; Chen and Liu 2024). As a result, the rhizosphere is a closely related plant-microbe system that is essential to the sustainability of agriculture and ecosystems. In recent decades, the possibility of using rhizospheric microbial populations to improve soil health, decrease the usage of chemical fertilizers, and increase plant growth and resistance has come to light (Wei et al. 2024). Studies on model plants and key crops like maize, wheat, and rice have demonstrated that the rhizosphere is home to beneficial microbes like phosphate solubilizers, nitrogen-fixing bacteria, and biocontrol agents (Kour et al. 2019; Imade and Babalola 2021; Di Benedetto et al. 2017; Ngalimat et al. 2021). Although many therapeutic plants are valued for their ability to produce secondary metabolites and are often cultivated in low-input systems, little is known about their rhizosphere microbiomes.

Long pepper, or *Piper longum* L., is a perennial climbing vine that is indigenous to South Asia and is a member of the Piperaceae family. Due to its medicinal qualities, which include antibacterial, anti-inflammatory, hepatoprotective, and anticancer actions, it is highly valued in ancient medical systems such as Ayurveda, Siddha, and Unani (Biswas et al. 2022). Its medical effectiveness is attributed to bioactive substances such piperine, piperdardine, and piperlongumine. There is a growing need for *Piper longum* to be grown using sustainable methods that preserve or improve its phytochemical content due

to the increased interest in natural products and herbal remedies around the world (Prasad et al. 2025). However, little is understood about the microbial communities in the rhizosphere that could affect its metabolite production, growth, and health.

Historically, the variety of soil microbiomes has been difficult for culture-based methods to fully capture because a large number of soil microorganisms are not culturable in standard laboratory settings. Microbial ecology has undergone a radical transformation thanks to the direct study of DNA made possible by developments in next-generation sequencing (NGS) technology, particularly metagenomic sequencing (Dubey et al. 2022; Raghav et al. 2024). Metagenomics provides information about the functional potential of microbial communities in addition to their taxonomic composition by identifying genes linked to the nutrient cycle, stress adaptation, and plant–microbe interactions (Kumar and Yadav 2024). In this study, we used high-throughput Illumina sequencing to conduct a comprehensive metagenomic analysis of the *P. longum* rhizosphere. Through gene annotation across major functional databases, this study sought to clarify the functional potential of the microbial communities residing in the rhizosphere of *P. longum* while also characterizing their taxonomic diversity. Finding dominant microbial taxa that may contribute to improving soil fertility, bolstering plant health, and maintaining ecosystem stability received special attention. This work lays the groundwork for future functional studies centered on microbiome-assisted strategies for sustainable cultivation, increased plant productivity, and improved phytochemical quality in medicinal plants by offering an integrated overview of the *P. longum* rhizosphere microbiome.

## **2. Material and Methods**

### **2.1 Soil sample collection for analysis**

The rhizospheric soil of the plants during the spike stage (November) was selected for the metagenomic study of the *P. longum* established in Herbal Garden, D.E.I, Agra. A total of three random replicates were considered for the study. The plants were uprooted, and the loosely attached soil was removed by vigorous shaking. The soil adhered tightly with the root surface was collected in a pre-sterilized container. A pooled sample was prepared for metagenomic analysis by mixing the rhizospheric soils of three replicates (Hou and Williams 2013; Znój et al. 2022). The samples for estimation of soil properties were collected and transported at room temperature whereas the samples for metagenomic analysis were collected and transported at 4°C and further kept at -20°C.

### **2.2 Soil parameter analysis**

These soil samples were mixed to form a composite of all collected samples. Samples were allowed to equilibrate with air for 2 hours in trays. The soil samples were dried and sieved with 2mm sieve. The physiochemical examination of the rhizospheric soil for its physical properties (pH, EC) and macronutrients (NPK and % organic carbon) was conducted according to the (Laishram et al. 2013; Nyoki et al. 2018).

## 2.3 Sample Preparation and DNA Extraction

The rhizospheric soil samples of *P. longum* were pooled and we sent to Xcleris Labs Ltd, Ahmedabad for further analysis. From the samples, the genomics DNA was isolated using the Xcelgen soil gDNA kit, following the manufacturer's protocol. The quality of the genomic DNA was assessed using 0.8% agarose gel electrophoresis. The gel was run for 30 mins at 110V. The DNA quantification was carried out using the Qubit® 2.0 Fluorometer. Samples exhibiting a single intact band and an A260/280 ratio between 1.8–2.0 were selected for library preparation.

## 2.4 Library Preparation and Sequencing

Paired-end sequencing libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina. Briefly, 200 ng of high-quality DNA was fragmented using a Covaris, end-repaired, and ligated with platform-specific adapters. Dual-indexed libraries were PCR-amplified using HiFi PCR Master Mix to ensure sufficient yield. The quality and average size distribution of the final libraries were assessed using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip. Sequencing was performed on the Illumina platform (2 × 150 bp chemistry) generating high-quality paired-end reads.

## 2.5 Bioinformatics Analysis

The raw reads were subjected to quality control to remove adapter sequences, ambiguous bases, and low-quality reads. High-quality paired-end reads were assembled *de novo* using the metaSPAdes assembler with default parameters optimized for metagenomic datasets. The assembly statistics, including the number of scaffolds, total scaffold length, N50, and maximum scaffold length, were calculated using in-house Perl scripts. Gene prediction was carried out using Prodigal (v2.6.3) in metagenome mode, which is designed to handle anonymous contigs from mixed microbial communities. These predicted gene sequences were used as input for taxonomic and functional analyses.

Taxonomic annotation was performed using Kaiju, a fast and sensitive metagenomic classifier that assigns taxonomy based on maximum exact matches at the protein level using the Burrows–Wheeler transform algorithm (Menzel et al., 2016). Predicted gene sequences were uploaded to the Kaiju web server (<http://kaiju.binf.ku.dk>) and analyzed with the following parameters: run mode = greedy, minimum match length = 11, minimum match score = 75, allowed mismatches = 5, and SEQ low-complexity filter = yes. Kaiju classified the sequences against the NCBI RefSeq microbial protein database, optionally including fungi and microbial eukaryotes.

Functional annotation of predicted genes from *Piper longum* was performed using COGNIZER (v0.9b), a comprehensive standalone pipeline that simultaneously assigns functional terms across multiple databases. The predicted gene sequences were analyzed to obtain Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Pfam, Gene Ontology (GO), and FIGfams annotations. COG analysis classified genes into functional categories involved in essential cellular processes such as amino acid transport and metabolism, transcription, replication, and energy

production. KEGG mapping associated the genes with pathway modules using KO identifiers, enabling the reconstruction of metabolic and signal transduction pathways. Pfam analysis identified conserved protein families and domains, providing insight into potential structural and functional properties. GO annotation categorized genes into biological process, molecular function, and cellular component classes, thereby facilitating the interpretation of gene roles in the microbial community. FIGfams annotation grouped genes into sets of functionally homologous proteins, allowing the identification of conserved metabolic capabilities across diverse taxa (Galperin et al. 2015). Collectively, these annotations provided a comprehensive understanding of the metabolic potential and functional diversity of the microbial community associated with *Piper longum*.

### 3. Results

The fertility status and suitability for microbial activity of the rhizospheric soil of *Piper longum* were evaluated by analyzing its physicochemical properties as shown in Table 1. With a pH of  $7.52 \pm 0.02$  and a neutral to slightly alkaline reaction, the soil showed ideal conditions for microbial growth and nutrient availability. A moderate amount of soluble salts that is within the permissible range for healthy plant growth is indicated by the electrical conductivity (EC), which was measured at  $639.21 \pm 0.04$  dS/m. While the levels of potassium ( $22.36 \pm 0.007$  kg/ha) and phosphorus ( $24.06 \pm 0.10$  kg/ha) demonstrated balanced nutrient availability necessary for plant metabolism and microbial functioning in the rhizosphere, the macronutrient composition showed a nitrogen content of  $310.19 \pm 0.03$  kg/ha, indicating moderate fertility. Low to medium organic matter was indicated by the organic carbon content of  $0.44 \pm 0.008\%$ . These soil parameters collectively show a biologically active, fairly fertile rhizosphere that supports the functional potential revealed by metagenomic profiling and is favorable for plant growth as well as the development of a metabolically diverse microbial community.

Table 1  
Physio-chemical properties of the  
rhizospheric soil of *Piper longum*.

| Soil Properties    |                   |
|--------------------|-------------------|
| pH                 | $7.52 \pm 0.02$   |
| EC (dS/m)          | $639.21 \pm 0.04$ |
| Nitrogen (kg/ha)   | $310.19 \pm 0.03$ |
| Phosphorus (kg/ha) | $24.06 \pm 0.10$  |
| Potassium (kg/ha)  | $22.36 \pm 0.007$ |
| Organic carbon (%) | $0.44 \pm 0.008$  |

To explore the rhizospheric bacterial community of *P. longum* metagenomic analysis was performed which yielded high-quality paired-end sequencing genomic DNA on the Illumina platform which generated 19,942,187 read pairs, yielding approximately (~ 5.92 Gb) of data with Q30 values exceeding

90%, which ensured high-confidence base calls. The quality-filtered reads were assembled *de novo* using metaSPAdes, resulting in the 97,432 scaffolds with a total length of 52.26 Mb, an average scaffold size of 536 bp, an N50 of 489 bp, and a maximum scaffold size of 44,108 bp as provided in Table 2. The assembly statistics provided indicated a comprehensive as well as well-represented metagenomic dataset with a sufficient coverage for downstream analysis. Further, genes were predicted using Prodigal (v2.6.3) which identified 45,876 protein-coding genes, with an average gene length of 334 bp and a total gene length of 15.36 Mb, providing a robust catalog of coding sequences for taxonomic and functional annotation as shown in Table 3.

Table 2  
*De novo* Assembly Statistics

| Assembly Elements          |          |
|----------------------------|----------|
| #Scaffolds                 | 97432    |
| Total scaffold length (bp) | 52264418 |
| Average Scaffold size (bp) | 536      |
| Scaffold N50               | 489      |
| Maximum scaffold size (bp) | 44108    |

Table 3  
Predicted Genes Statistics

| Description            |          |
|------------------------|----------|
| #Genes                 | 45876    |
| Total gene size (bp)   | 15362571 |
| Average gene size (bp) | 334      |
| Max scaffold size (bp) | 3699     |

Taxonomic classification was performed using Kaiju software, unraveling the microbial community associated with *Piper longum* which was found to be quite diverse; however, it was strongly dominated by the phylum Proteobacteria, that accounted for 14,015 hits (~ 42% of classified genes). Other major phyla that constituted the majority of the bacterial population in the *P. longum* rhizosphere with includes, Actinobacteria (4555 hits) which accounted for 13.9% of the total classified genes, Thaumarchaeota (13.16%), Chloroflexi (8.21%), Acidobacteria (7.28%), Candidatus rokubacteria (2.47%), and Nitrospirae (1.92%) whereas the least found phylum was that of Candidatus Fraserbacteria, Candidatus Roizmanbacteria and Thermodesulfobacteria (4 hits or 0.012% of relative abundance) as shown in Fig. 1. By digging into the finer taxonomic resolution, the most abundant class was found to be of Gammaproteobacteria, with relative abundance of 26.01%, followed by Betaproteobacteria with 17.09%, Actinobacteria with 12.02% and Nitrososphaeria 11.03%, respectively (Fig. 2). Similarly, the order level of

classification revealed that Nitrososphaerales have a relative abundance of 15.82% was the dominant order, which was mirrored by the dominance of Nitrososphaeraceae (20.28%) at the family level (Figs. 3 & 4). At the genus level, *Nitrososphaera* was found to be the most abundant with relative abundance of 23.58% followed by *Acinetobacter* (13.11%), *Rubrobacter* (6.36%) and *Nitrospira* (5.06%), while the most abundant species detected was *Candidatus Nitrososphaera gargensis* with relative abundance of 11.21%, followed by *Acidobacteria bacterium* (9.74%) (Figs. 5 & 6). The preponderance of nitrifying archaea, such as *Nitrososphaera*, is indicative of the existence of an active ammonia-oxidizing microbial community. This community may be responsible for nitrogen cycling in the rhizosphere of *Piper longum*, indicating a community structure that is not only very diverse but also functionally relevant.

A thorough understanding of the metabolic capacity of the *Piper longum* microbiome was obtained by the functional analysis of the predicted genes using COGNIZER (v0.9b). The COG analysis identified 23,705 terms, with amino acid transport and metabolism accounting for the highest prevalence (2649 terms) which was followed by prediction of general function (2488 terms), carbohydrate transport and metabolism (2100 terms), and energy generation and conversion (1900 terms) (Fig. 7). This distribution emphasizes the microbial community's metabolic activity, especially in the areas of energy metabolism and nutrient cycling.

The metagenome sequencing from the current work has been submitted to the NCBI website under the accession number PRJNA600362. A variety of protein databases, including KEGG, Pfam, GO, COG, and FIG, were used to annotate the datasets with functional hierarchy information for the functional predictions (Fig. 8). The GO database produced the greatest number of hits, followed by the KEGG and Pfam databases. The COG functional analysis identified 23705 terms, and the KEGG annotation assigned 27,446 terms to 3594 KO classes, with the majority (54.6%) pertaining to metabolic pathways such as amino acid biosynthesis, carbohydrate metabolism, and energy production pathways. The next most prevalent functional category was environmental information processing (25.5%), followed by genetic information processing (12.7%), showing the existence of genes involved in two-component systems, ABC transporters, and transcriptional control as shown in (Fig. 9). The annotation of Pfam domains revealed 24,583 functional domains in 2930 distinct Pfam families, indicating a great variety of structural and catalytic proteins. Further clustering of 15,148 words into 4518 functionally homologous groupings using FIGfam analysis suggests conserved functionality across several taxa.

The Gene Ontology (GO) annotation generated 33,522 words over 952 functional categories, with the largest representation in biological processes such as metabolic activity, stimulus response, and cellular functions. A substantial number of genes were classified as molecular function categories such as catalytic activity and binding, as well as cellular component categories related to membrane proteins and intracellular activities. These findings collectively indicate that the microbiome associated with *Piper longum* is physiologically flexible, with a focus on nutrition mobilization, energy metabolism, and environmental adaptation. These findings collectively show that the microbiome of *P. longum* is rich and functionally diversified, dominated by ammonia-oxidizing archaea and Proteobacteria, and that metabolic pathways linked to nitrogen, carbohydrate, and amino acid cycling are strongly enriched. This

functional potential highlights the ecological relevance of the *P. longum* microbiome in maintaining plant growth, improving the availability of nutrients, and possibly affecting the manufacture of secondary metabolites.

## Discussion

A strong relationship between soil fertility parameters and the functional competence of the microbial community in the *Piper longum* rhizosphere is highlighted by the combination of metagenomic insights and soil nutrient profiling. Plant-microbe symbiosis and microbially mediated nutrient cycling are supported by the moderate organic carbon content and balanced nutrient composition. The ecological and biotechnological significance of the *P. longum* microbiome is highlighted by these interactions, which not only maintain soil health but may also have an impact on the biosynthesis of pharmacologically significant compounds like piperine. The present research offers novel insights into the taxonomic constitution, functional potential, and ecological importance of the related microbial community by conducting a thorough metagenomic analysis of the *Piper longum* microbiome. The number of scaffolds and a N50 value of 489 bp demonstrate the high-quality and outstanding coverage of the dataset produced by high-throughput Illumina sequencing in tandem with *de novo* assembly. The assembly and gene prediction outcomes were similar to those reported for other medicinal plants, including *Tribulus terrestris*, *Piper nigrum*, *Calatropis procera*, where plant-associated microbial communities have been investigated using comparable sequencing depths (Rahimlou et al. 2025; Vaz et al. 2025; Li et al. 2016; Ramadan et al. 2021).

As demonstrated by the taxonomic profiling, Proteobacteria clearly dominated the rhizosphere and endophytic communities in medicinal plants. Proteobacteria are often the most abundant phylum because of their diverse metabolic capacities and capacity to colonize plant tissues (Vincze et al. 2024). The high prevalence of betaproteobacteria and gammaproteobacteria among the Proteobacteria indicates a metabolically active community that can react quickly to changes in nutrient levels in the rhizosphere (Manapure et al. 2022). Given that these ammonia-oxidizing archaea are important participants in the nitrification process and aid in the conversion of ammonia to nitrite, a crucial stage in the nitrogen cycle, the identification of Nitrososphaerales and the dominance of *Candidatus Nitrososphaera gargensis* seems particularly significant (Xiang et al. 2023; Clark et al. 2021). Their presence strengthens the possibility that the rhizospheric zone of *P. longum* is a nitrogen cycling hotspot, which could improve the intake and growth of nitrogen by plant. The robust vegetative development and the generation of bioactive metabolites under natural conditions may be partially explained by these microbial interactions. The metabolic adaptability of the *P. longum* microbiome was further highlighted by functional annotation. A microbiome that is actively involved in nutrient mobilization is indicated by the enrichment of COG categories linked to energy production, carbohydrate metabolism, and amino acid transport and metabolism (Sefrji et al. 2025; Xu et al. 2021). Through the synthesis of precursors for secondary metabolites, such as alkaloids like piperine, amino acid metabolism affects plant health in addition to being essential for microbial proliferation (Narayanan et al. 2022).

With notable presence in pathways related to nitrogen, sulfur, and carbon cycle, the KEGG pathway analysis showed that over half of the annotated genes fell under the metabolic category. These results are consistent with recent research on various microbiomes of medicinal plants, which emphasize the role of environmental information processing and nutrient cycling in promoting plant development and metabolite biosynthesis (Semenzato and Fani 2024; Singh et al. 2022). Remarkably, genes linked to stress response and xenobiotic degradation were also found, indicating that the *Piper longum* microbiome is capable of assisting the plant in overcoming abiotic stress and possibly enhancing resistance in harsh environmental circumstances (Pandey et al. 2023; Chaudhary et al. 2022).

Additionally, our findings offer a useful resource for comprehending interactions between microbes and plants in relation to the generation of secondary metabolites. Since the availability of nitrogen is a known regulator of alkaloid production, the presence of microbial genes involved in nitrogen cycling, phenylpropanoid metabolism, and amino acid biosynthesis may have an indirect effect on piperine biosynthesis (Gao et al. 2022). It may be possible to determine whether particular microbial species actively control the piperine biosynthesis pathway by future functional investigations, such as transcriptomics and metabolomics. Targeted enrichment of these species may increase the efficiency of nitrogen utilization in *Piper longum* culture, and the discovery of nitrogen-transforming archaea like *Nitrososphaera* opens up possibilities for the development of biofertilizer (Li et al. 2023). Overall, this study creates a baseline microbiome profile for *Piper longum*, exposing a metabolically active and taxonomically varied microbial community that may play roles in secondary metabolism, stress adaptation, and nutrient cycling. These discoveries broaden our knowledge of the ecological functions of microorganisms linked to plants and point to potential uses in precision farming, where piperine productivity could be increased through microbiome engineering.

## Conclusion

Plant health and soil nutrient dynamics depend on the taxonomically diverse and functionally rich microbial community found in the rhizosphere of *Piper longum*, according to metagenomic research. The abundance of Proteobacteria and ammonia-oxidizing archaea such as *Nitrososphaera* and *Candidatus Nitrososphaera gargensis* indicates that the rhizosphere is likely to be an active site of nitrogen transformation. Functional gene annotation demonstrated a significant representation of metabolic pathways associated with energy synthesis, amino acid transport, and environmental adaptation, as well as a microbiome that is precisely regulated to support plant development in a variety of soil conditions. Together, the data suggest that the rhizospheric microbiota of *P. longum* is important for the cycling of nutrients and may indirectly affect the production of compounds that are relevant to pharmacology, such as piperine. This study highlights the importance of using beneficial microbial consortia for sustainable cultivation and increased phytochemical production, while also offering a basic understanding of the *P. longum* microbiome. Future multi-omics methods that combine transcriptome and metabolomic studies will be essential to identify possible taxa for the development of bioinoculants in medicinal plant agriculture as well as to pinpoint the exact microbiological processes underlying the production of secondary metabolites.

# Declarations

## Competing interests

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

## Consent to participate

Not applicable to this manuscript.

## Consent for publication

We give our consent and ensure that the publisher has the author's permission to publish the research article in their Journal.

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# Author Contribution

**\*\*M.P.\*\*** Conceptualization **\*\*S. M.\*\*** Writing original draft **\*\*R. R.\*\*** Review and Supervision, S.K. and A.C. Editing. All authors reviewed the results and the manuscript.

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# Data Availability

The metagenome sequencing from the current work has been submitted to the NCBI website under the accession number PRJNA600362

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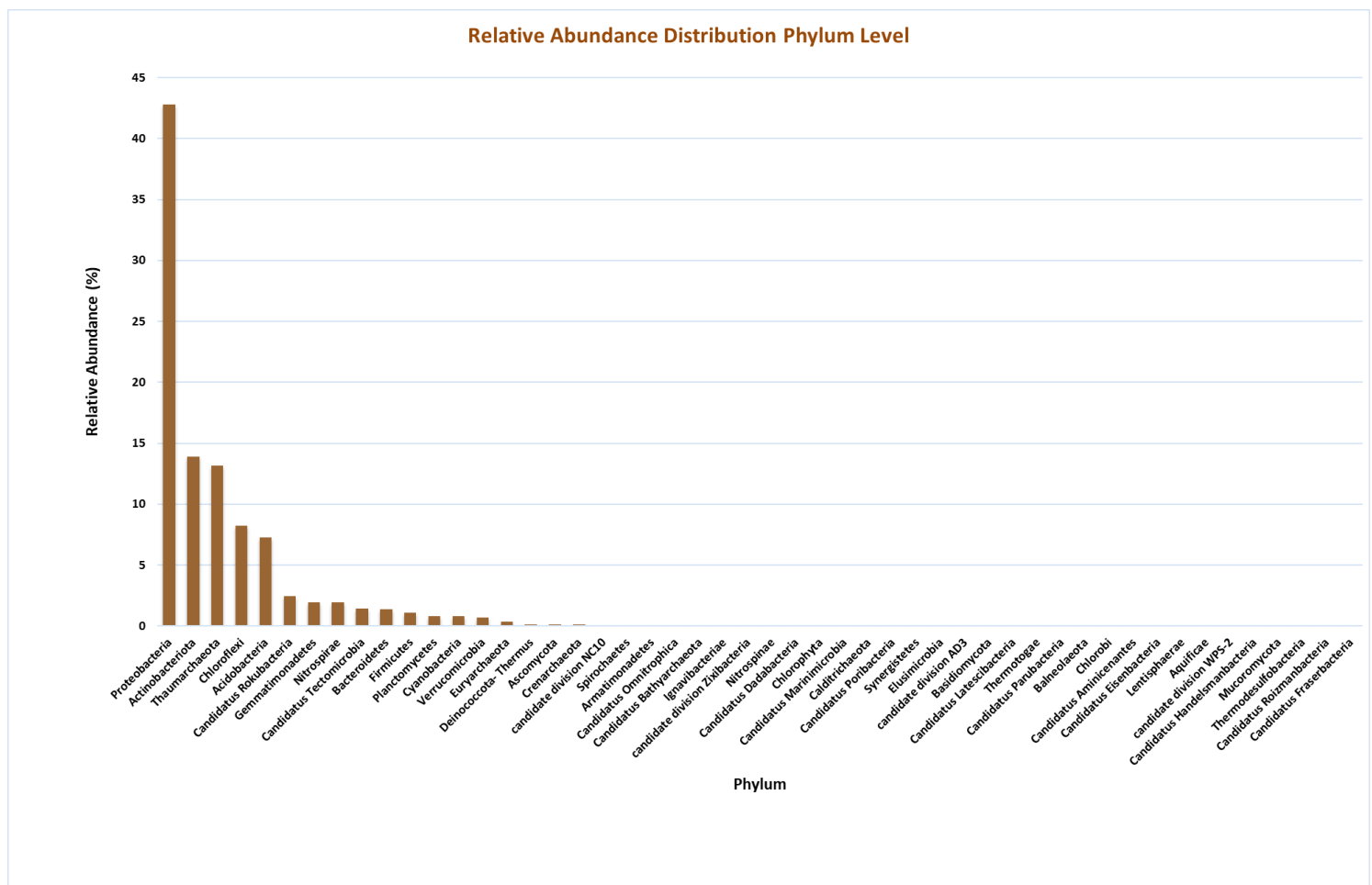
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## Figures



**Figure 1**

Bar chart showing the taxonomic abundance of sample *Piper longum* at phylum level. From the figure, it can be inferred that Proteobacteria is the most abundant phylum followed by Actinobacteria.

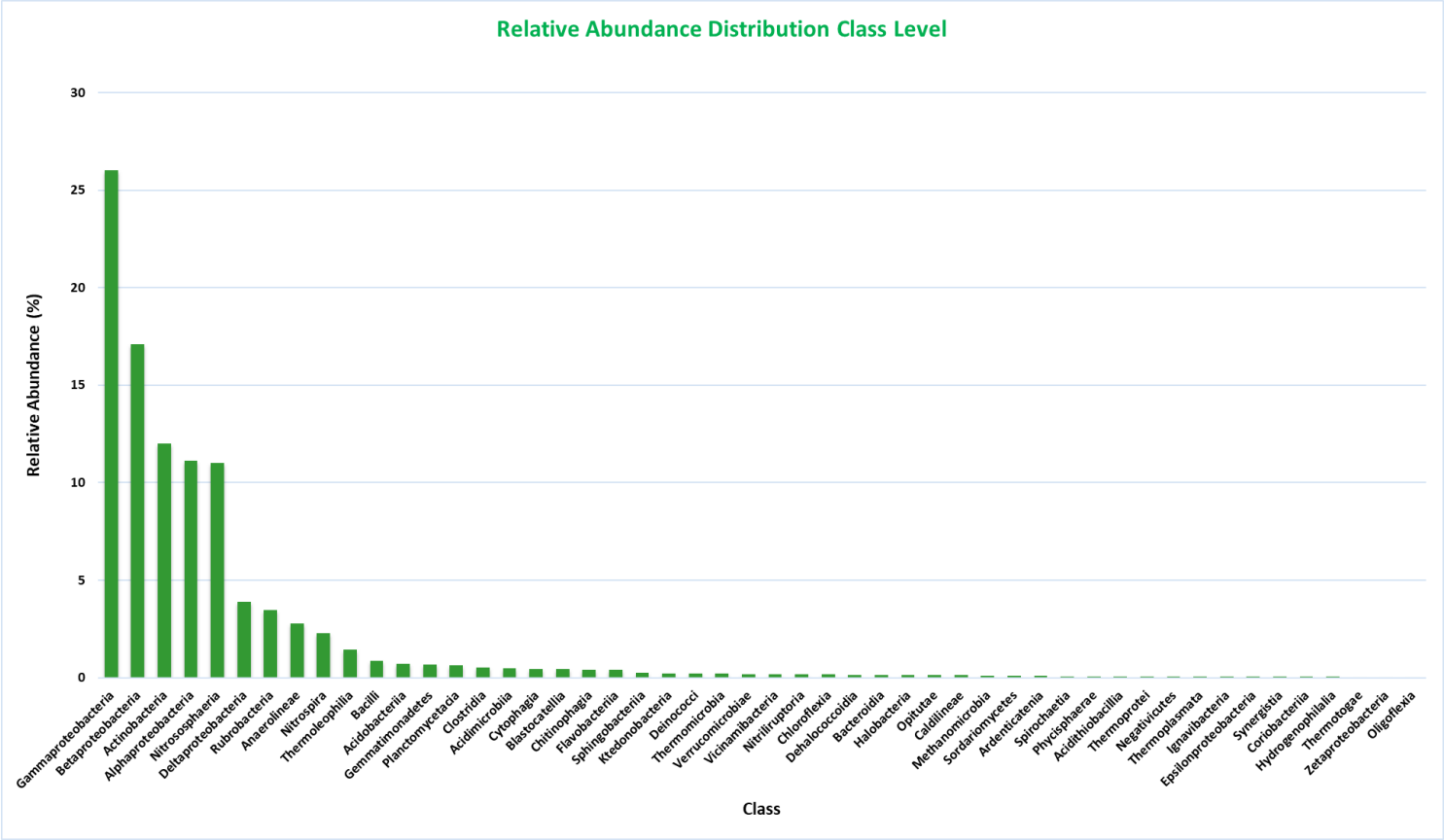


Figure 2

Bar chart showing the taxonomic abundance of *Piper longum* Sample at class level. From the figure it can be inferred that Gammaproteobacteria is the most abundant class followed by Betaproteobacteria.

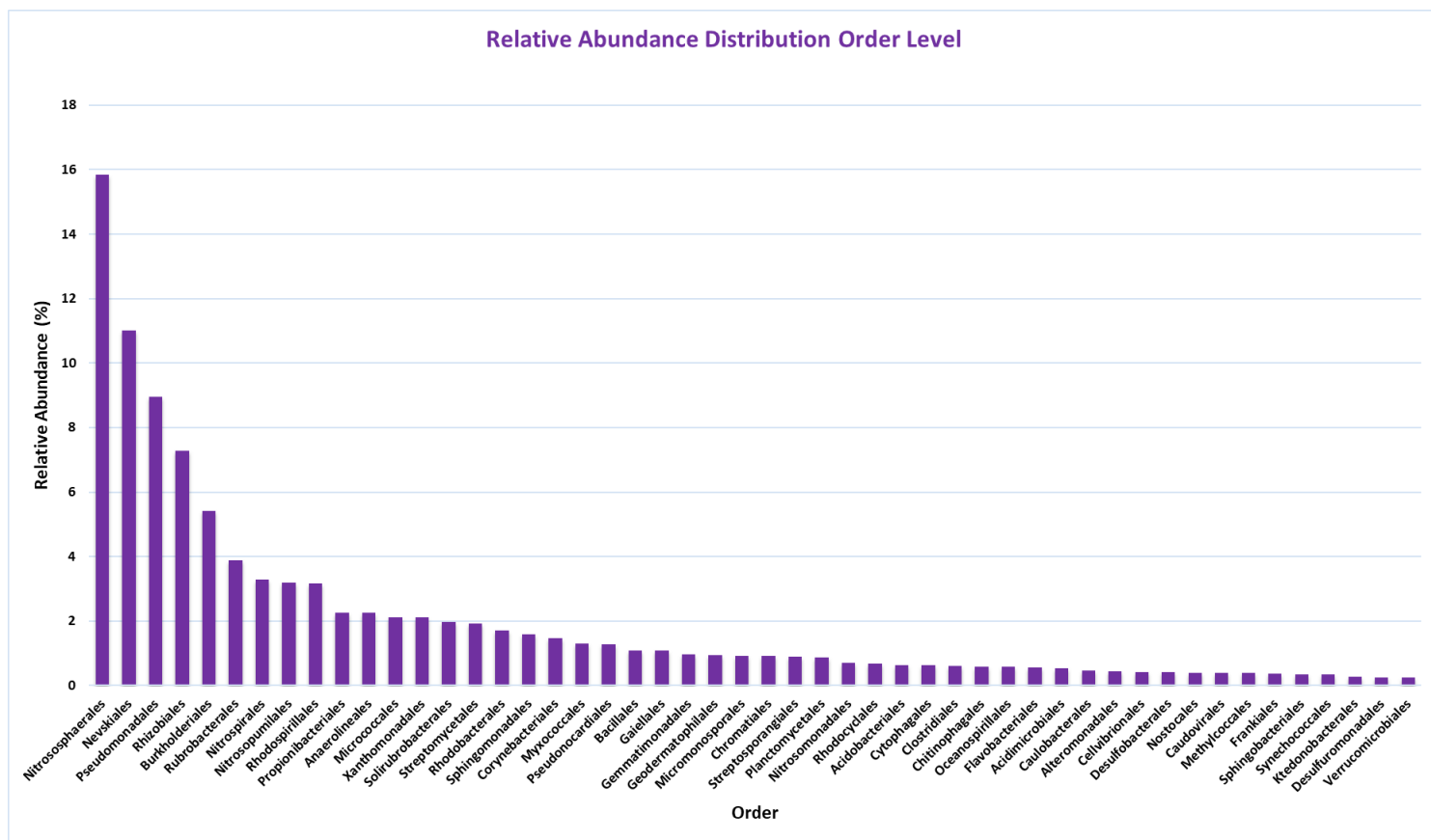


Figure 3

Bar chart showing the taxonomic abundance of sample *Piper longum* at order level. From the figure, it can be inferred that Nitrososphaerales is the most abundant order followed by Nevskiales.

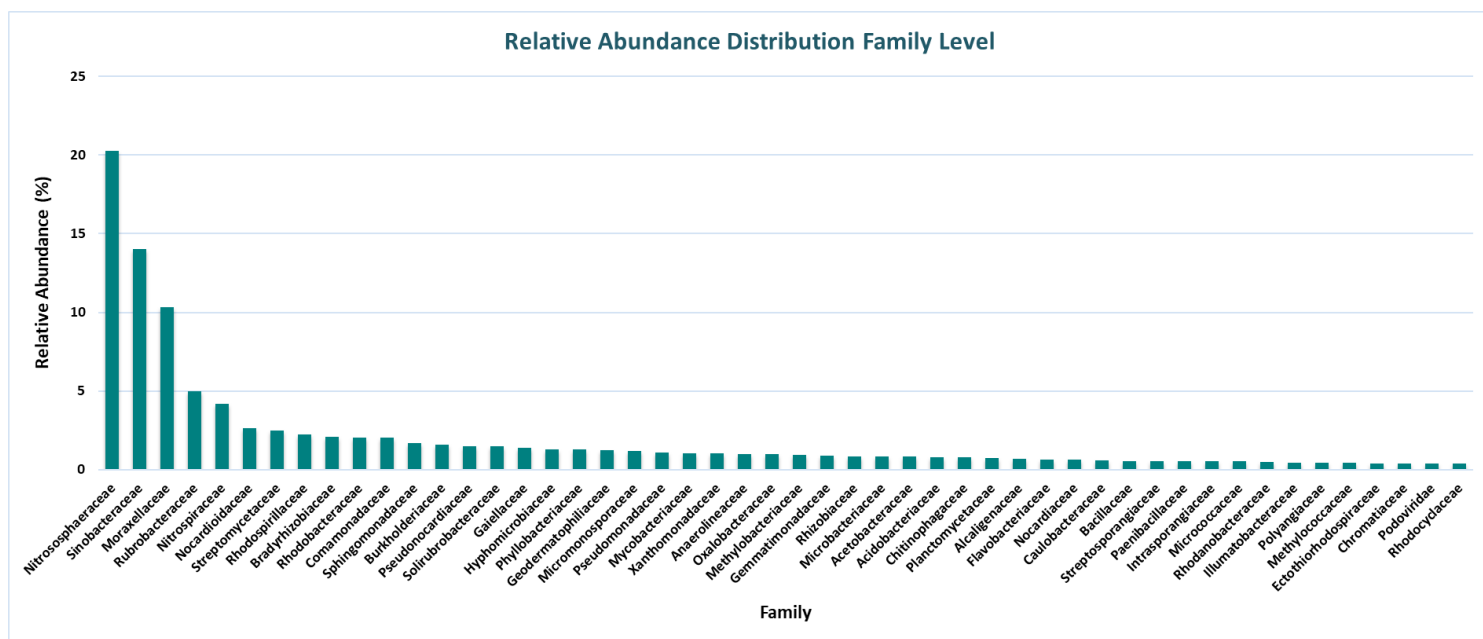
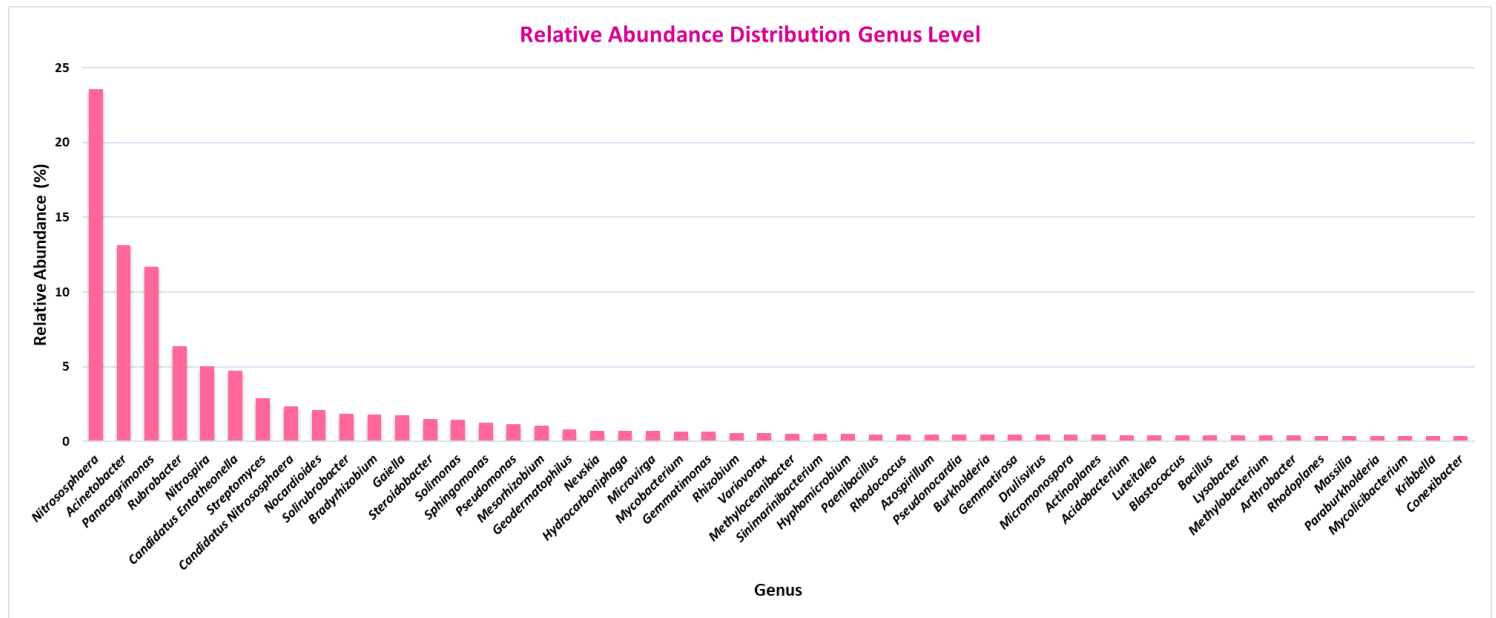


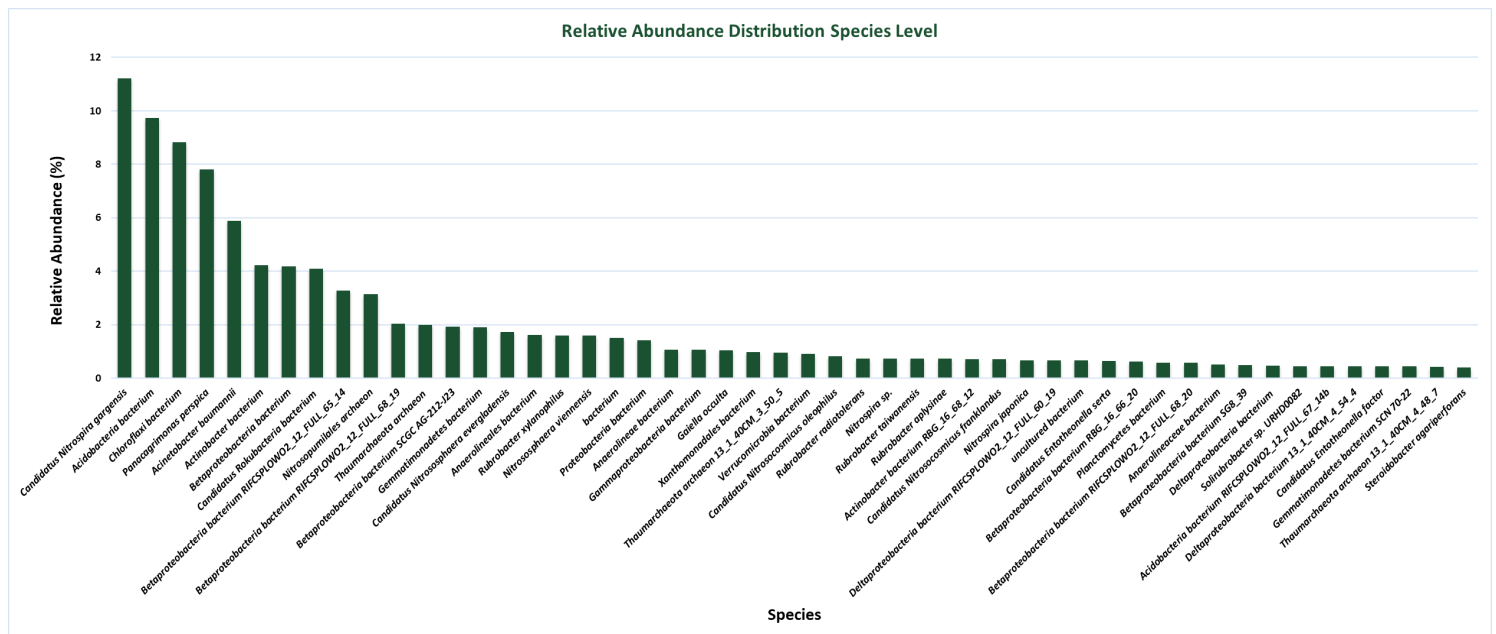
Figure 4

Bar chart showing the taxonomic abundance of sample *Piper longum* at family level. From the figure, it can be inferred that Nitrososphaeraceae is the most abundant family followed by Sinobacteraceae.



### Figure 5

Bar chart showing the taxonomic abundance of sample *Piper longum* at genus level. From the figure, it can be inferred that Nitrososphaera is the most abundant genus followed by Acinetobacter.



### Figure 6

Bar chart showing the taxonomic abundance of sample *Piper longum* at species level. From the figure, it can be inferred that *Candidatus Nitrososphaera gargensis* is the most abundant species followed by *Acidobacteria bacterium*.

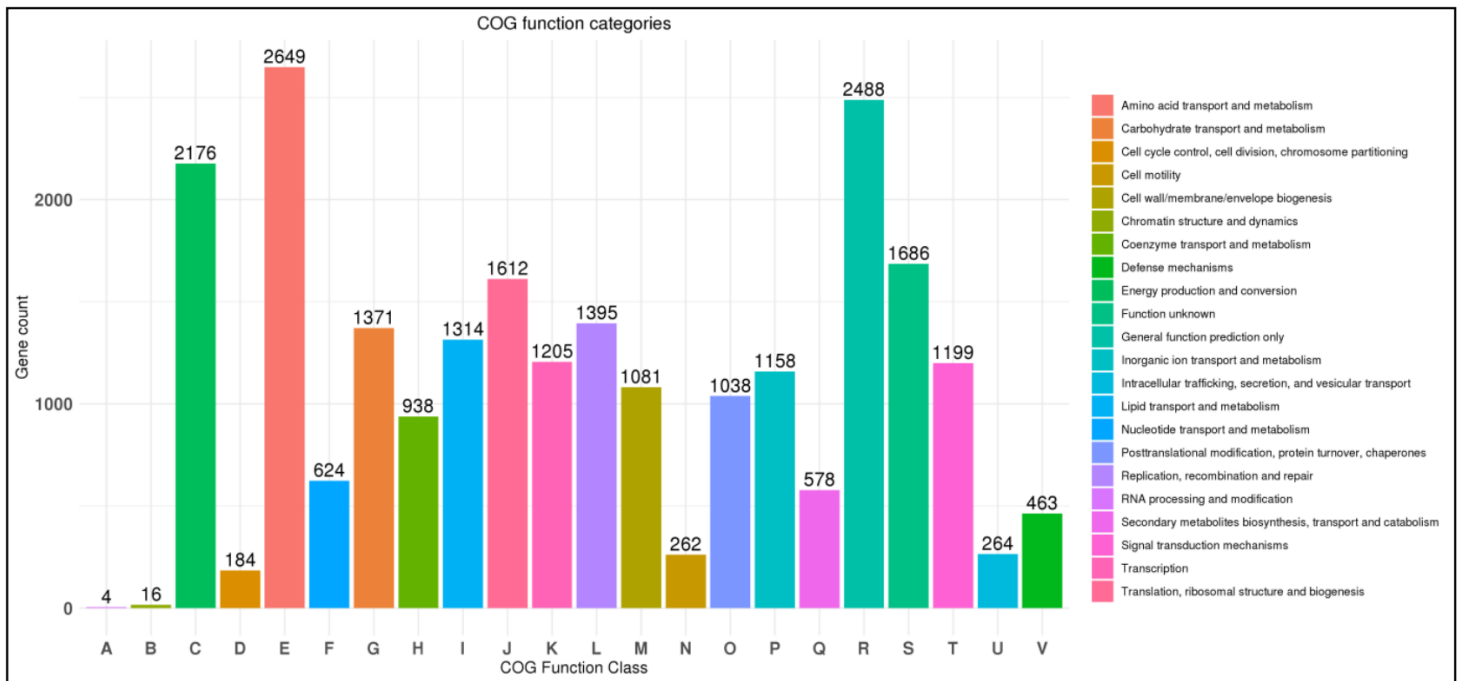


Figure 7

COG Functional Category Hits Distribution of Sample *Piper longum*.

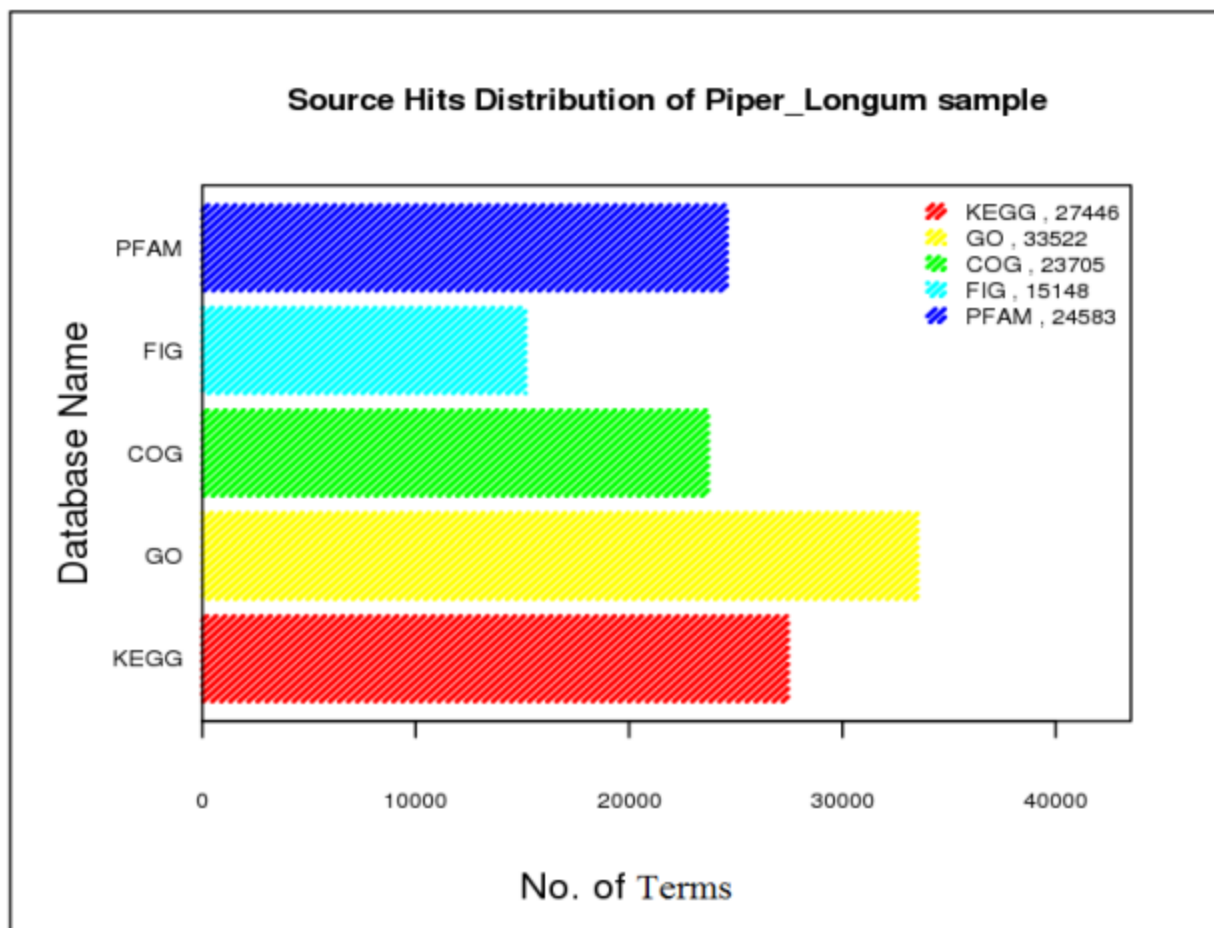


Figure 8

Source hits distribution of Sample *Piper longum*. Each number indicated represents the number of terms annotated against the respective databases. Maximum number of hits was observed in GO, followed by KEGG and PFAM.

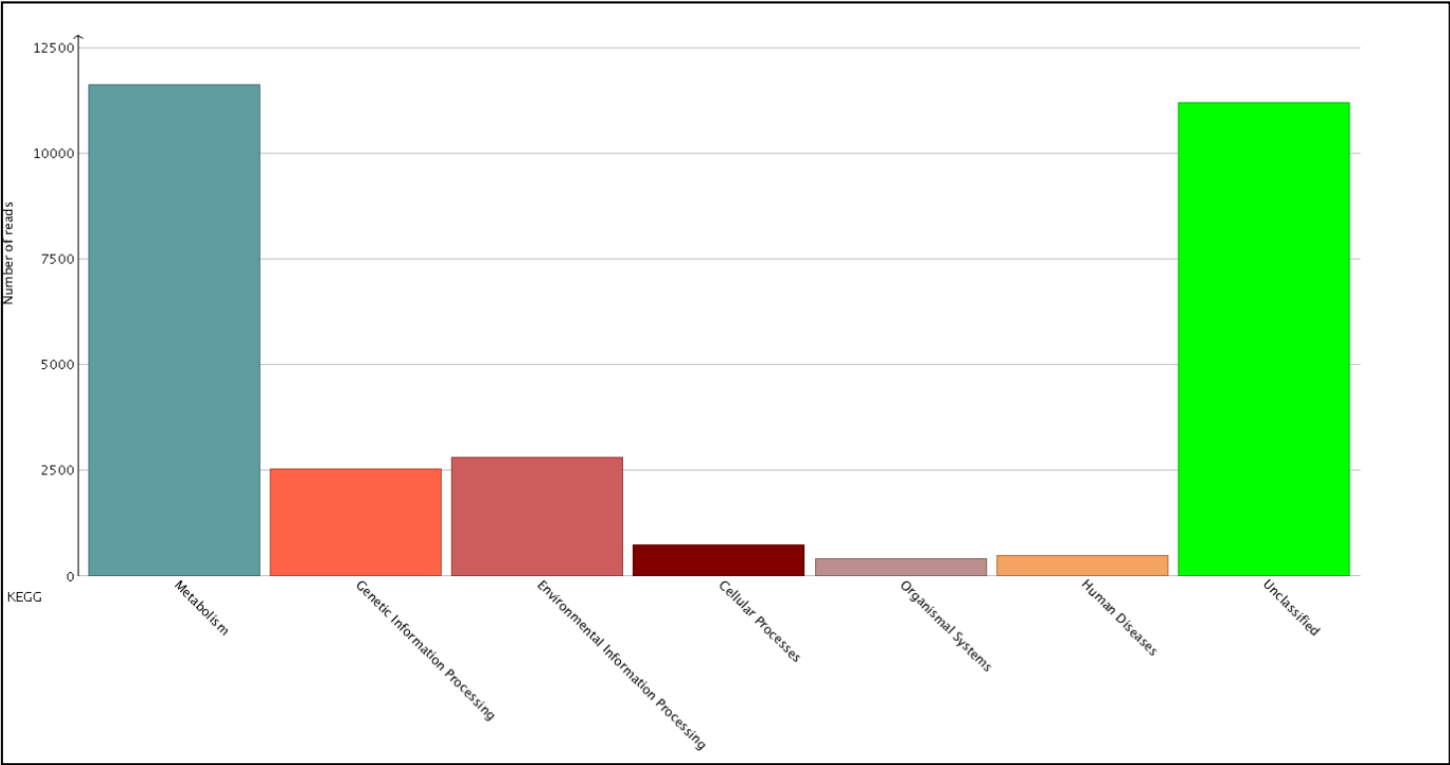


Figure 9

KO Functional Category Hits Distribution of *Piper longum*.