Metagenomic Outlooks of Microbial Dynamics Influenced by Organic Manure in Tea Garden Soils of North Bengal, India

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Abstract

Soil being the most heterogeneous and complex microbial habitat on earth exceeds the quantity of inhabiting microbial communities than other environments. Next-generation sequencing (NGS) based metagenomics provides us directs access to the uncultivated genomes. In this study, we targeted two very popular tea gardens of Darjeeling hills- Makaibari (Mak) and Castleton (Cas). The main difference between them is the type of manure they use. Mak is solely an organic tea garden using all organic manure and fertilizers whereas Cas uses inorganic pesticides and fertilizers. The main aim was to compare the effect of organic manure over chemical fertilizers on the soil microbiome as well as the health of tea garden workers. We have performed the 16s metagenomics analysis based on V3-V4 region. Downstream bioinformatics analysis including Reverse Ecology was performed. We found that the overall microbial diversity is higher in Mak rather than Cas. Moreover, the use of organic manure has reduced the population of pathogenic bacteria in Mak soil when compared to Cas soil thus having an indirect effect on the heath of the tea garden workers. From the observations made through the metagenomics analysis of Mak and Cas soil samples we may propose that the application of organic manure supports the population of good bacteria in the soil which eventually can have a better impact on the tea garden workers.

Introduction

Soil being the most heterogeneous and complex microbial habitat on earth exceeds the quantity of inhabiting microbial communities than other environments. Some phylogenetic surveys on soil ecosystems made evident that the number of prokaryotic species present in a specific soil sample is far more than the known cultured prokaryotes (Daniel, 2005). Metagenomic analyses endow extensive information about the structure, composition, and predicted gene functions of varied environmental assemblages. Soil metagenomics comprising of DNA isolation from the soil along with production and screening of clone libraries have proved to be an authenticated measure to explore the cultivation-independent genetic reservoir of soil microbial communities. The unmatched diversity of soils promise sustained exploration of diverse industrial, agricultural and environmental agents in the future. Each environment has its unique challenges to metagenomics investigation requiring explicitly designed approaches considering both biotic and physicochemical factors (Kakirde et al. 2010, Nesme et al. 2016).

The successful application of soil metagenomics demands high-quality DNA extraction, purification along with cloning methods for the anticipated downstream bioinformatics analyses (Kakirde et al. 2010). The most vital technical deliberation in metagenomics includes obtaining a sufficient yield of high-quality pure DNA in lieu of the targeted microbes within an environmental sample. The average insert size of a clone library or the length of sequence read for a high-throughput sequencing approach is also crucial. An appropriate metagenomics screening strategy should be adapted to address the specific question(s) of interest (Kakirde et al. 2010,).

Despite all these obstacles, next-generation sequencing (NGS) based metagenomics provides us direct access to the uncultivated microbes. The high throughput sequencing technology has equipped the field of microbiology with new phyla, class, genera, species as well as functional microbial genes (Nesme et al. 2016). The ability of metagenomics can answer numerous what, how who and why questions. For example, which type of soil hosts which kind of microbes? How the microbes interact with each other and also with the surrounding environment? Do they act synergistically or antagonistically? How the microbes act on changing environments and so on.

India is one of the major biodiversity hubs on this planet comprise of several different types of soils. This versatility ranges from snow soil to desert sand, from beach sands to most fertile riparian soil. The geographical location of this country has blessed it with a large area of agriculture friendly fertile soil with high yielding capacity. Paddy, wheat, green vegetables are common to grow in most of the Indian soils. However, one specific beverage crop that originally came to this country from outside and got very well adapted to the North-Eastern part of India is tea. Darjeeling tea has become world-famous for its brilliant aroma and color. Modern science has recognized the impact of the microbial population on the yielding capacity of the soil. Thus, it is now well known that soil along with its microbial communities can modulate not only the environment beneath the earth's crust but also above it including the crops that are grown in the soil along with other higher-order organisms and humans dependent on that soil in particular means.

In this study, we targeted two very popular tea gardens of Darjeeling hills- Makaibari (Mak) and Casselton (Cas). The main difference between them is the type of manure they use. Mak is solely an organic tea garden using all organic manure and fertilizers whereas Cas uses inorganic pesticides and fertilizers. Our main aim is to identify different sets of microbes that are present in these two tea garden soils using NGS-based metagenomic sequencing and to venture how the microbial population of both these tea gardens is affected by the types of fertilizers being used. We tried to detect how the microbes are interacting among them, through complementation (synergy) or competition (antagonistic property) and how microbes are impacting the quality of the soil and tea are grown on it. Also, we will try to understand whether these soil microbes affect the health quality of the tea garden workers or not.

Materials And Methods

Field of study, sample collection and soil testing

We have chosen two popular tea gardens from the Darjeeling hill region- Makaibari (26.8716° N, 88.2678° E) and Casselton (26.8659° N, 88.2777° E). The distance between these two tea gardens is only 12 min (4.0 km) via NH110 and they were on the same side of the hill. Makaibari is solely an organic tea using organic fertilizers and manure whereas; Casselton uses chemical pesticides and chemical fertilizers. Soil samples were randomly collected from the rhizosphere region of tea plants. Debris from the samples like roots, pebbles, etc. was removed by hand. Soil texture was assessed by the field method. The moisture percentage of soil samples was determined from the difference in weight of freshly collected and oven-

dried soil samples. The clean air-dried samples were passed through a sieve and crushed with mortar and pestle. Soil pH, Electrical conductivity, and Loss of ignition were estimated following the protocol of Baruah and Barthakur, 1997). Other important parameters like organic carbon (Walkey and Black, 1974), total soil Nitrogen (Jackson, 1973) phosphorus as phosphate (Baruah and Barthakur, 1997; Jackson, 1973; Bray and Kurtz, 1945) and potassium (Chapman and Pratt, 1961), sulphur were determined during soil analysis. The level of micronutrients was qualitatively assessed by micronutrient kit. Information regarding the health of the tea garden workers were collected from a survey based approach. The persons directly associated with the tea workers health of both organic and inorganic manure based tea gardens were interviewed for collecting information regarding the present health scenario of those gardens.

DNA isolation

Soil DNA was isolated using rhizosphere soil of Makaibari and Casselton tea garden. Before initiating the isolation, 2 g of respective soil (three replicates per sample) was mixed with 4 ml of 1X Tris-EDTA buffer followed by proper vortexing (4-5 min) in 50 ml Oakridge tube. Before cell lysis, 150 µl of lysozyme (50 mg/ml), 100 µl of Proteinase K (20 mg/ml) and 600 µl of freshly prepared 10% SDS were mixed with the soil samples and the sample incubated at 37ºC for 90 min with gentle shaking every 15 min interval. After incubation, 1 ml 5M sodium chloride, 1.6 ml CTAB/NaCl was mixed with respective solution and further incubated at 65ºC for 30 min with occasional mixing in between to release the DNA from microbial cells. The supernatant containing microbial DNA was extracted with chloroform–isoamyl alcohol (24:1, v/v) and collected in a new tube after centrifugation at 6000 rpm for 15 min at room temperature. The aqueous phase containing DNA was precipitated with 0.6 volumes of cold isopropanol and 0.1 volumes of 3M sodium acetate followed by 2 h incubation at -20ºC. The DNA pellets were obtained by centrifugation at 10,000 rpm for 30 min at 4ºC, washed with cold 70% ethanol, and dissolved in 100 µl of 1X Tris-EDTA buffer. To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/280 nm (DNA / protein) were determined and the DNA was sent for 16s Metagenomics amplicon sequencing (V3-V4) to Genotypic Technology Pvt. Ltd.3. 16s Metagenomics analysis

This analysis has been performed using the Parallel-META pipeline (version 3.5; Jing, et al., 2017).

Taxonomic and functional profiling

Parallel-MEA 3 initially constructs Hidden Markov Models (HMM) using all bacterial 16S rRNA sequences of SILVA (version 123; Pruesse et al. 2007) and forecasts the 16S rRNA gene fragments in metagenomic shotgun samples from both the forward sequences and reversed complementary sequences by HMMER (version 3.1, e-value < 1e-5). Then Parallel-META 3 extracts all the 16S rRNA fragments from metagenomic shotgun sequences for profiling. All 16S rRNA gene sequences (either extracted from shotgun sequences or 16S rRNA amplicon reads) are aligned to the Parallel-META 3 reference database by Bowtie2 (Langmead et al., 2012) for OTU picking, taxonomical annotation and phylogeny construction. The reference 16S rRNA sequences are from a customized database that integrates GreenGenes (version 13-8, sequence similarity on 97% level; DeSantis et al., 2006) with RDP and SILVA consensus taxonomy annotation (assigned by BLASTN with e-value < 1e-30 and similarity >97%), which

raised the proportion of annotated sequences at the genus level from 35.8% to 81.5%. The phylogenetic architecture of all reference sequences is built by FastTree (Price et al., 2010). Since the 16S rRNA gene copy number varies greatly among different bacterial species, Parallel-META 3 also calculates the precise relative abundance of each organism by 16S rRNA copy number calibration using IMG database (Markowitz et al., 2012). Besides, considering that the uneven sequencing depth (number of sequences) among multiple samples may introduce bias in detecting diversity patterns (Koren et al., 2013), an optional sequence rarefaction for sequencing depth normalization at the OTU level is provided after the taxonomic profiling.

For prediction and annotation of the functional profile, Parallel-META 3 re-implements the PICRUSt (Langille et al., 2013) algorithm using the KEGG database (Kanehisa et al., 2012) to estimate all the functional genes harbored in a microbiota using 16S rRNA gene OTUs. The functional genes are annotated by KO (KEGG Ontology) and KEGG pathway. Parallel-META 3 also measures the prediction accuracy by the NSTI (Nearest Sequenced Taxon Index) value (Langille et al., 2013), which is calculated by the sum of distances between OTUs and their nearest individually sequenced relatives in the phylogenetic architecture. (Jing, et al., 2017)

After taxonomical and functional profiling, Parallel-META 3calculatedthe sequence counts and relative abundances (normalized into 0–100%) for all OTUs, and estimates the same information for annotated taxa from the phylum level to the genus level, as well as the genes and the pathways. Such data is structured into tables compatible for further analysis in Parallel-META 3 and also suitable for manual examination by users. (Jing, et al., 2017)

The α diversity evaluation and statistics

Parallel-META 3 estimates α diversity describing the inner complexity of each microbiota sample. This process generates rarefaction curves of α diversity based on observed OTU number and Shannon index to determine the adequacy of the sequencing depth. The rarefaction performs a series of random sequence selection on different sequencing depth with bootstrap (default = 20), and α diversity in the curves is calculated by the mean sequence count of each OTU among the bootstrapping procedures. Then the influence of each environmental factor on α diversity is quantitatively evaluated by multivariate statistical analysis with Shannon index, Simpson index and CHAO1 index. (Jing, et al., 2017)

Parallel-META 3 examines β diversity of multiple microbiota samples based on their pair-wise similarity matrix to discover the patterns of organism/gene sharing and variation among samples. The quantitative similarity between each sample pair is computed by Meta-Storms (Su et al., 2012) algorithm, which considers both the relative abundance of OTUs existent in two samples and the distances among OTUs in the phylogenetic architecture. The β diversity evaluation includes unsupervised hierarchical clustering, supervised clustering using PCA (Principal Component Analysis), PCoA (Principal Co-ordinate Analysis) and multivariate statistical analysis that quantitatively evaluates the correlation between environmental factors and the sample similarities. (Jing, et al., 2017)

Biomarker discovery

Parallel-META 3 can categorize key organisms or functional genes that are highly correlated with the variations of the habitats or other types of metadata. Organisms or genes with significant differences among microbial community samples were firstly chosen using Kruskal-Wallis or Wilcoxon rank-sum test as candidate makers, and these candidate makers are then ranked based on their contribution to the differentiation among samples using the Random Forest algorithm. (Jing, et al., 2017)

Construction of microbial interaction network

The microbial interaction network is constructed to explore co-occurrence and co-exclusion patterns of organisms or functional genes across microbial community samples. In the interaction network, each node represents a single organism (or gene), and nodes are connected by links that represent their correlation coefficient of abundance variation among multiple samples (Faust, et al., 2012). Subsequently, Parallel-META 3 exhibits the global pattern among numerous samples by network's topological characters such as number of nodes, number of isolated islands, diameter, density, centralization and radius. (Jing, et al., 2017)

16S rRNA sequencing

The 16S rRNA sequencing was done using Illumina™ Nextseq platform. The raw reads qualities were checked using FastQC. Mean quality score for each base, per sequence quality score, per sequence GC contents and per base, N contents are calculated. Adapter contamination was present in less than 0.1% reads; therefore, adapter trimming was not performed.

Downstream analysis of the metagenomic sequencing

MG-RAST server was used to analyze the high quality reads from each sample for taxonomic profiling and metagenomic data. We intended to do an *in-silico* ecological study based on reverse ecology analysis. Hence we selected the type strain from each of the genus identified in both the samples and downloaded their metabolic information from the KEGG database. RevEcoR, an R-based package (Cao et al. 2016) was used to assess the reverse ecological interactions among each set of microbes in terms of competition and complementation.

Results

Physiochemical properties of Mak and Cas soil

Both the tea garden considered for this study had loam soil. Mak soil was light, friable loam with porous subsoil. This soil type is preferred for tea due to free percolation of water. The Cas soil was clay type. The low pH of both the soils indicated towards the acidic nature of the soil which is good for tea. Results of soil physicochemical analysis are shown in the table (Supplementary Table 1). The results were compared with soil physicochemical standards recommended by the Tea Board of India. The clayey soils

of the tea plantations have low pH, sulphur but high organic carbon, organic matter, total nitrogen and P2O5. K2O was optimum in soil collected from Mak but high in soil from Cas. Micronutrients like Boron, Manganese, Zinc and Copper were low while Molybdenum was moderate in both the plantations. Iron was optimum in Mak but low in Cas. The soil types of the tea gardens were not largely different. However the difference arose in the fertilizes used by these two tea gardens. Mak is practicing with the organic manure filled with vermicompost, bio-fertilizers and organic manure while Cas is totally dependent upon the inorganic manure and pestiscides for maintaining Sodium(Na): Pottasium ratio, weed control along with pest and disease management.

Primary data summary

The paired-end reads from Mak and Cas soils gave 56% and 55% average GC respectively. There were 0.2 M sequences for each read of Cas with 67.95% duplication value and 0.3M sequences for each read of Mak with 72% duplication value. FastQC report revealed good quality reads indicating successful metagenomic sequencing.

MAK is more populated than CAS with good bacteria leading to a stable ecotype model

The taxonomic abundance profiling identified the microbial abundance from phylum to genus level (Fig 1). It was found that both soils shared a set of bacteria however; their relative abundance was not the same. The major microbial phyla identified in both soil samples were- *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Choloflexi*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* (Fig 1a, 1b). *Cyanobacteria* and *Gemmatimonadetes* were present in Mak constituting 1.32% and 1.24 % of the total respectively whereas; they constituted less than 0.5% in Cas soil. Some unidentified sequences were also present however, they were not considered for further analysis. Most interestingly, a distinct pattern was observed between the two soil samples when microbes were identified at the genus level. For instance, the relative abundance of the pathogenic microbial population was found to be more in CAS than MAK (Fig 1c). The abundance profile of pathogenic microbes like *Burkholderia, Campylobacter*, and *Bacillus* were much higher in Cas than in Mak. Moreover, the abundance of *Mycobacterium* was also more in Cas than Mak. Along with those mentioned genera, the presence of *Candidatus Solibacter, Vibrio, Candidatus Koribacter, Peptoniphilus, Peptoniphilus, Clostridium, Gemmatimonas* were found in both Cas and Mak. Their abundance was not very high in any of the soil samples. *Bradyrhizobium, Microcoleus, Acidimicrobium, Streptacidiphilus, Achromobacter, Gemmata and Frankia* were solely present in Mak but not in Cas however, *Dehalogenimonas, Saccharopolyspora, Isosphaera, Acidobacterium, Chthoniobacter, Ktedonobacter, Thermotoga* were solely present in Cas but not in Mak. This indicated the differential bacterial population among Cas and Mak.

To find the species diversity between the two soil samples α diversity of both samples were exploited. Alpha (α) diversity is a direct measure of mean species diversity of habitat and a higher α diversity value indicates more diversity. The α diversity value of Cas was 48.69 and for Mak it was 56.62 pointing to more species richness in Mak. A Rare-fraction curve that allows us to calculate the species richness from a given number of individual samples was further implemented to support our aforementioned

hypothesis. A common pattern of this curve is, it grows rapidly at first due to the most common species present in the samples and gradually becomes a plateau as the rarest species remain to be sampled. In CAS, the curve started to get a plateau state at species count 1400 (Fig 2a) wherein MAK the stage came at species count 3000 (Fig 2b). Hence, it is evident from taxonomical abundance profiling, α diversity and rare-fraction curve analysis that, Mak is more ecologically diverse with a higher microbial population rather than Cas.

Functional profiling on both samples revealed Metabolism along with Information storage and processing (ISP) as major functional categories in which both Mak and Cas microbial population are involved in. In Mak, microbes were also associated with cellular processing and signaling (CPS) procedure which was totally absent in Cas sample indicating a more complex biological network among Mak microbial population rather than Cas. Reverse ecology analysis was implemented to get a birds-eye view on the complex microbial interaction and signaling network going on within Mak and Cas soil samples. Reverse ecology analysis evident a sharp complementation pattern among the microbial population of both Cas and Mak. The complementation index clearly indicated a strong biological association with peaceful resource sharing among the microbial population of both Cas and Mak (data available on request).

Soil microbial population of tea gardens impacting the health of garden workers

Cas microflora shows the predominance of pathogenic flora, *Bacillus*, which includes the food borne pathogenic species *Bacillus cereus.* This is known to cause mild gastroenteritis to severe and sometimes source of hepatitis, fatal diarrhea, typhoid fever and dysentery (Bottone, 2010). Tea garden workers are also prone to infections of the respiratory tract (e.g., *Legionella*, typical mycobacteria). The other pathogenic microbes include *Naegleria fowleri, Burkholderia pseudomallei* which causes infection in the skin and brain (Inglis & Sagripanti, 2006). The microaerophilic and capnophilic *Campylobacter spp*. are some of the most important causes of acute gastroenteritis worldwide (Frost 2001) which shows prevalence in Cas field. On the other hand, frequency of beneficial soil microflora such as *Bifidobacteria* in Mak indicates the positive impact of organic farming. The probiotic agent *Bifidobacteria* has been commercially exploited due to their associated health benefits and has GRAS (Generally Recognized As Safe) status (Picard et al., 2005). Apart from these, the use of *Bifidobacteria* to treat various gastrointestinal disorders has also been reported. *B. longum* subsp. *infantis* CECT 7210 and *B. breve* K-110 was found to inhibit *Rotavirus*, which causes sporadic diarrhea in infants (Bae et al., 2002; Chenoll et al., 2015).

Discussion

The overall soil physicochemical properties were alike as they belong to the same eco-geographical region and altitudinal level. The overall bacterial diversity was more in Mak than Cas. Moreover, Cas population contained more pathogenic genus than Mak. This clearly indicated a positive effect of organic manure in comparison to inorganic/ chemical fertilisers. The complementation value (obtained from

reverse ecology analysis) among the Mak population was higher than Cas population. This may indicate a stable ecotype model (SEM) (Shapiro and Polz, 2015) persisting in Mak where the main carbon source of soil is organic manure. It's a well-known fact that, fertilizers have a direct impact on soil microbial population playing a pivotal role in both biogeochemical cycling and ecological processes (Li et al. 2017). Certain microbial taxa display ecological coherence in response to environmental variables. Based on substrate preference and life strategies, those microbes can be grouped into r-selected or k- selected categories. However, it's difficult to gain such knowledge at a lower taxonomic level (genus or species level). It has been documented previously that, continuous exposure of fertilization (both organic and inorganic) leads to the addition of a specific category of carbon (C) and nitrogen (N) source to the soil. Over a period, a set of bacteria, capable to handle those specific C and N sources will proliferate in that agricultural field. This practice in the long run is good for providing agroecosystem stability. Organic manures are composed of different decomposing materials hence contain diverse C and N sources. On contrary, chemical fertilizers are always well defined with their source of C and N (Li et al. 2017). As a result, it may well be predicted that a field exposed to long-term organic manure will house a more versatile microbial population utilizing various kinds of nutrient sources than a field exposed to defined inorganic manure. Makaibari tea garden is popular for using organic manure since its inception whereas, Castleton uses inorganic fertilizers. These differential practices are thus, playing a major role in the microbial population between these two tea gardens.

The microbial diversity and interactions in the soil are extremely complex (Bhattacharyya et al., 2014) which is affected by various parameters such as microbiological components with multifaceted metabolic activities, root exudates, and several other biotic and abiotic factors. The soil microflora has a direct effect on human health which is evident from the health status of workers. Most of the tea garden workers are prone to respiratory ailments such as tuberculosis and skin disorders. Extensive use of chemical fertilizers and pesticides results in the degradation of soil and water bodies. Agricultural chemical inputs gain access into human body systems through three major means: (1) oral ingestion, (ii) infiltration through the skin, and (iii) breathing (Roy Chowdhury et al., 2014). Systemic toxicity can occur by persistent exposure to these chemicals. This may manifest in the form of neurological, gastrointestinal, renal, or hepatic toxicity. Since the vast majority of workers in the tea plantation are women, concerns have centered on the potential reproductive hazards of chemical exposure and their impact on pregnant women, nursing mothers, and their children. Moreover, the chemical residues affect the quality of tea thereby reducing its economic potential.

Conclusion

Soil metagenomics has become an important tool in studying the unculturable microorganisms present in a specific niche. In this present study, we did 16s metagenomics of Makaibari (Mak) and Castleton (Cas) tea gardens from Darjeeling, India. The main difference between them is, Mak is an organic manure-based tea garden whereas Cas use chemical fertilizers. Metagenomics revealed higher bacterial diversity in Mak than Cas. The pathogenic bacterial population was more in Cas than Mak indicating the positive feedback effect of organic manure on the bacterial population of soil. We investigated

interactions among the identified genus from both Mak and Cas. A stable ecotype model was evident in Mak where microbes were showing synergistic effect (complementation) whereas; in Cas soil competition was more among the bacterial population reveling volatility of ecosystem. Finally, the number of human pathogens was more in Cas than Mak which supported the better tea garden worker health report in Mak than Cas. Thus, this study indicates that the organic fertilizers have a positive effect on the soil microbial population and human health in a broader aspect.

Declarations

Acknowledgments

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Author contributions

ARS conceived the idea and did the experimental design. PK, SC, GS, MB collected and prepared samples for metagenomics. MB and PK and SB did the soil analysis. ARS, SB and IS did the bioinformatics analysis. IS, PK, SC, GS, SB, ARS wrote and corrected the manuscript and generated all figures and tables. All authors have reviewed the manuscript.

Conflict of interest

The authors declare that the research paper was written in the absence of any commercial or financial relationships that could be construed as real or potential conflict of interest.

Availability of data and materials

All related supplementary data are included in this paper.

Animal Issues

No animal or human were treated as sample in this study.

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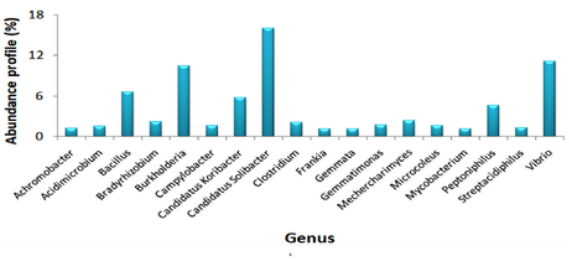
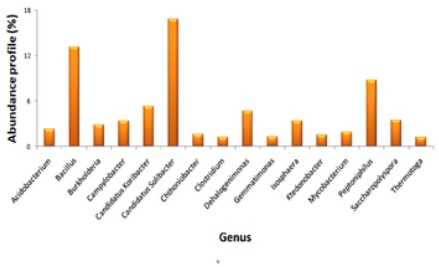
Figures





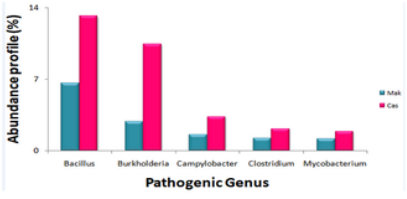


Figure 1

(a) Abundance profile of Makaibari soil sample (b) Abundance profile of Casselton soil sample (c) Differential distribution of the pathogenic genus between Mak and Cas.

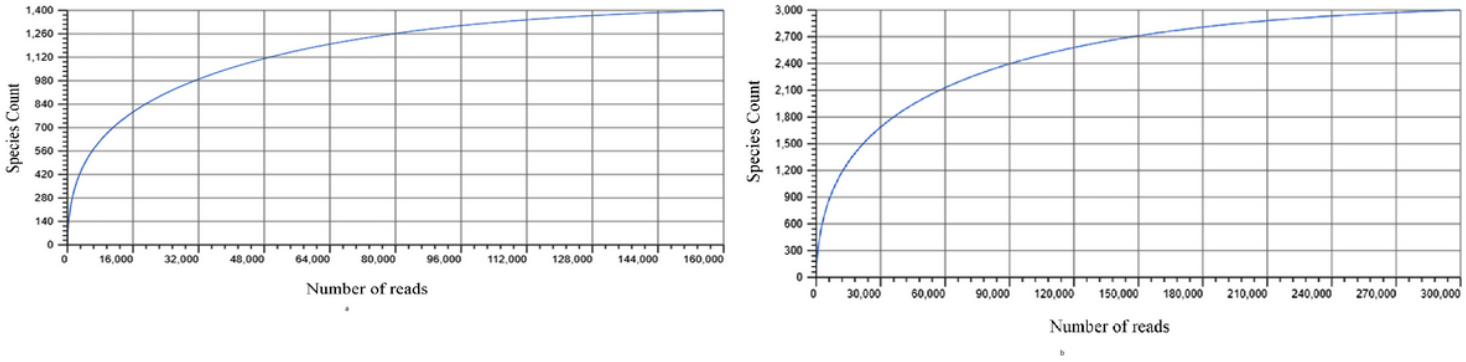


Figure 2

(a) Rare fraction curve for Cas soil sample (b) Rare fraction curve for Mak soil sample

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable2.docx