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ORIGINAL PAPER

Metagenomic outlooks of microbial dynamics influenced by organic manure in tea garden soils of North Bengal, India

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Abstract

Soil microbial diversity consisted of both culturable and non-culturable microbes. The cultivated microbes can be identified by conventional microbiological processes. However, that is not possible for the non-culturable ones. In those cases, next-generation sequencing (NGS)-based metagenomics become useful. In this study, we targeted two very popular tea gardens of Darjeeling hills—Makaibari (Mak) and Castleton (Cas). The main difference between these two study areas 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 microbiomes. We have performed the 16 s metagenomics analysis based on the V3–V4 region. Downstream bioinformatics analysis including reverse ecology was performed. We found that the overall microbial diversity is higher in Mak compared to Cas. Moreover, the use of organic manure has reduced the population of pathogenic bacteria in Mak soil when compared to Cas soil. From the observations made through the metagenomics analysis of Mak and Cas soil samples, we may conclude that the application of organic manure supports the population of good bacteria in the soil which may eventually impact the tea garden workers' health.

Keywords Tea garden · Soil metagenomics · Fertilizers · Stable ecotype model

Introduction

The microbial diversity of soil is enormous. Both culturable and non-culturable microbes enrich the soil microbial population. 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). Conventional techniques for isolating and identifying the culturable microbes are not enough to study the overall diversity of soil microorganisms since it will exclude the considerable portion of non-culturables. Fortunately, with the advancement of metagenomic analysis, we can now gain a holistic idea about the microbial diversity of a specific soil sample.

Metagenomic analyses endow extensive information about the structure, composition, and predicted gene functions of varied environmental assemblages. Hence, 16 s metagenomic analysis based on the V3–V4 region has become a popular practice to unveil the effect of biotic and physicochemical factors on the overall microbial population of soil (Kakirde et al. 2010; Nesme et al. 2016).

There are some aspects of Metagenomics which are crucial to get the most accurate and relevant data from a sample. Extraction and purification of high-quality DNA is the major pre-requisite of any successful metagenomic study. The average insert size or the length of sequence reads for a high-throughput sequencing approach is also crucial. An appropriate metagenomics screening strategy should be adapted

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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 comprising 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 explore how the microbial population of both these tea gardens might be affected by the types of fertilizers being used. In this study, we deciphered the overall microbial population of both Makaibari (Mak) and Casselton (Cas) soil with special reference to their interaction among each other in terms of both complementation (synergy) or competition (antagonistic property). Along with that, we also tried to investigate whether the microbial population of two selected tea gardens may somehow affect the overall 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 valley 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 1962), sulphur was 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 was incubated at 37 ºC for 90 min with gentle shaking every 15 min interval. After incubation, 1 ml 5 M sodium chloride, 1.6 ml CTAB/NaCl was mixed with respective solution and was 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 3 M sodium acetate followed by 2 h incubation at − 20 ºC. The DNA pellets were obtained by centrifugation at 10,000 rpm for 30 min at 4 ºC, 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 16 s Metagenomics amplicon sequencing (V3–V4) to Genotypic Technology Pvt. Ltd.3.

PCR amplification of V3–V4 region of 16 s gene

About 40 ng of extracted DNA was used for amplification along with 10 pM of each primer (5' AGAGTTTGATG-MTGGCTCAG3' primer for forward sequence and 5' TTA CCGCGGCMGCSGGCAC3' primer for reversed sequence). The initial denaturation temperature was set as 95 °C. The denaturation was done for 15 s. Annealing was done at 60 °C for 15 s followed by elongation at 72 °C for 2 min. Final extension was done at 72 °C for 10 min. The final PCR product was stored at 4 °C. The amplified 16 s PCR Product was purified and subjected to GEL Check and Nanodrop QC. The Nano Drop readings of 260/280 at an approximate value of 1.8 to 2 were used to determine the DNA's quality.

Overall sequencing procedure

The amplicons from both samples were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantitated using Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2 × 300PE v3 sequencing kit.

Processing of metagenomics data

Raw data QC was done using FASTQC and MULTIQC, followed by trimming of adapters and low-quality reads by TRIMGALORE. The trimmed reads were further taken for processing which includes merging of paired-end reads chimeria removal and OUT abundance calculation and estimation correction—this was achieved by Parallel-META pipeline. This workflow enabled highly accurate investigations at genus level. The databases used were SILVA (https://www.arb-silva.de/) / GREENGENES (https://greengenes.secondgenome.com/) and NCBI (https://www.ncbi.nlm.nih.gov/). Each read was classified based on % coverage and identity. A schematic diagram of the overall 16 s metagenomics process has been diagrammatically represented in Supplementary Fig. 1.

Reverse ecology analysis

Reverse ecology analysis is a simple yet effective way to study the interaction among microbes present in specific sample. This analysis considers both competition and complementation to assess the overall interaction among microbes.

The present metagenomic study identified microbes up-to genus level. We used a cut-off value of 200 sequence count i.e. if the count is less than 200 for a specific genus, we simply did not consider it. This was done purely to have a manageable amount of out-put data and to remove the possibilities of false-positives. We have used the same cut-off value for all further analysis in this study.

The whole genome sequences of the type strains from the identified genus (with count > 200) of both Mak and Cas were considered for reverse ecology analysis. Their KEGG Ontology (KO) information were retrieved from KAAS database. The KO information was fed into RevEcoR, an R-based package (Cao et al. 2016) to compute the competition and complementation indices among the studied strains.

Results

Physicochemical 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 fertilizers 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 pesticides for maintaining sodium (Na):pottasium (K) ratio, weed control along with pest and disease management.

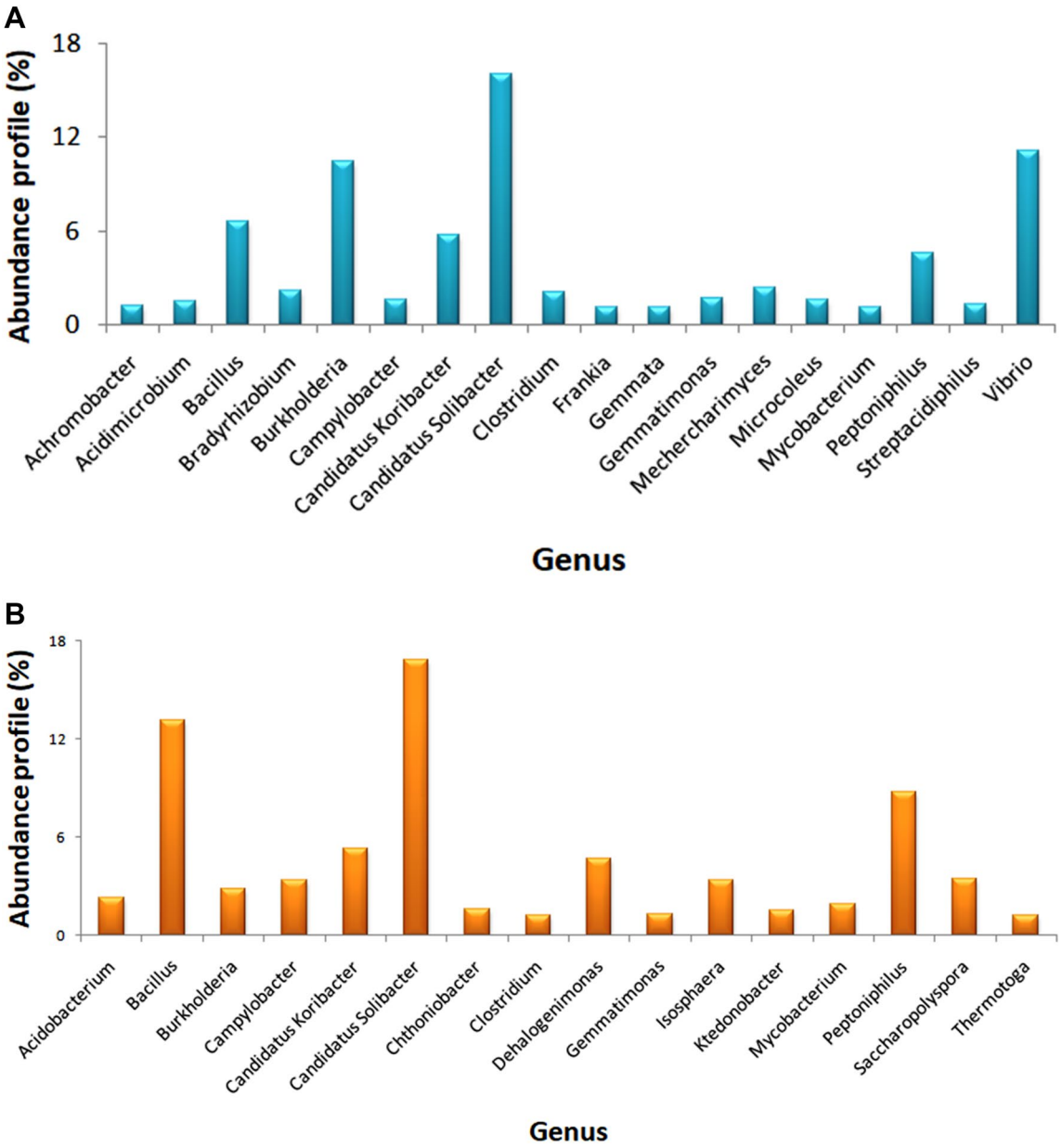

Fig. 1 (**a**) Microbial abundance profile of Mak. (**b**) Microbial abundance profile of Cas (color figure online)

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 0.3 M sequences for each read of Mak with 72% duplication value. The read lengths were 256 bp and 205 bp for forward and reverse sequences of Cas samples. Mak forward and reverse sequence lengths were 258 bp and 189 bp, respectively. FastQC report revealed good quality reads indicating successful metagenomic sequencing. The 16 s metagenomics data for Mak and Cas has been submitted in NCBI SRA under the BioSample accession: SAMN21875714 with BioProject ID: PRJNA766783.

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. It was found that both soils shared a large set of bacteria however, their relative abundance was not the same. For instance, *Cyanobacteria* and *Gemmatimonadetes* were present in Mak constituting 1.32% and 1.24% of the total microbial population,

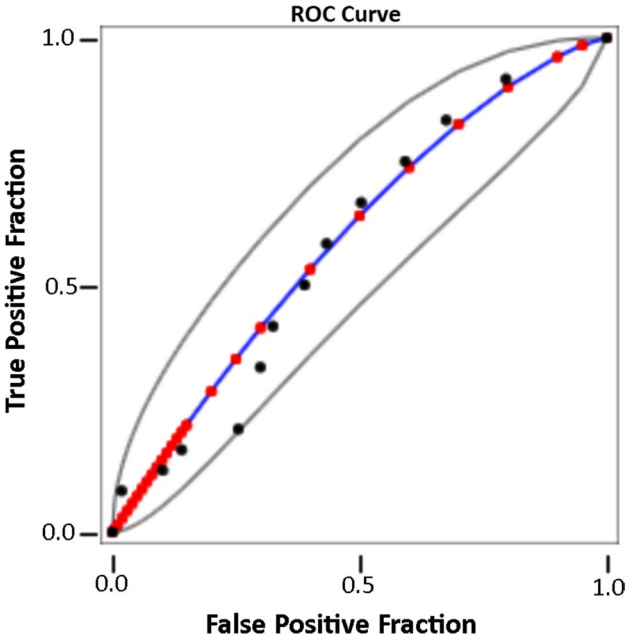

Fig. 2 The receiver operating characteristic (ROC) plot analysis among Mak and Cas (color figure online)

respectively, whereas they constituted less than 0.5% in Cas soil. The major microbial phyla identified in both soil samples were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Choloflexi*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* (Fig. 1a, 1b). We did the one-way ANOVA test, however, found no significant difference among the overall microbial population of Mak and Cas. The f ratio value was found to be 0.00589 and p value was 0.939465 and it was not significant even at $p < 0.01$. The receiver operating characteristic (ROC) curve (http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html) was plotted based on the overall microbial diversity among Mak and Cas (Fig. 2). The analysis gave fitted AUC (area under ROC) as 0.59 and empiric AUC was 0.585 suggesting less discrimination between the overall microbial population of Mak and Cas.

However, a distinct pattern was observed between the two soil samples when only pathogenic microbes were considered. For instance, the relative abundance of the pathogenic microbial population was found to be more in Cas than Mak (Fig. 3). 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, 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. When the one-way ANOVA test was performed the f ratio came to be 7.75 with p value 0.010285 and the result was significant at $p < 0.05$ (at $p < 0.01$ the difference was non-significant). A PCA plot based on the pathogenic microbial population also supported the ANOVA results where Mak and Cas were placed in two different quadrants of the PCA plot (Fig. 4).

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. 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. 5a) wherein Mak the stage came at species count 3000 (Fig. 5b). 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.

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. The reverse ecology analysis revealed that, the complementation index for Mak microbial population varied from 0.79–0.97 and that of Cas was 0.68–0.85. The competition index for both the samples were considerably low (0.21–0.39 for Mak and 0.32–0.41 for Cas). The differences between complementation and competition in both Mak and Cas were statistically significant (t test at $p < 0.001$). Moreover, the complementation among Mak population was more than Cas population ($p < 0.001$). This also supports that, the Mak microbial population has formed a more stable ecotype model than Cas which indicates a better natural selection forming and maintaining specific genetic clusters.

Discussion

Microbial population of Mak formed a stable ecotype model

We have found a clear picture about the microbial diversity of both studied tea gardens. Here are the major findings we

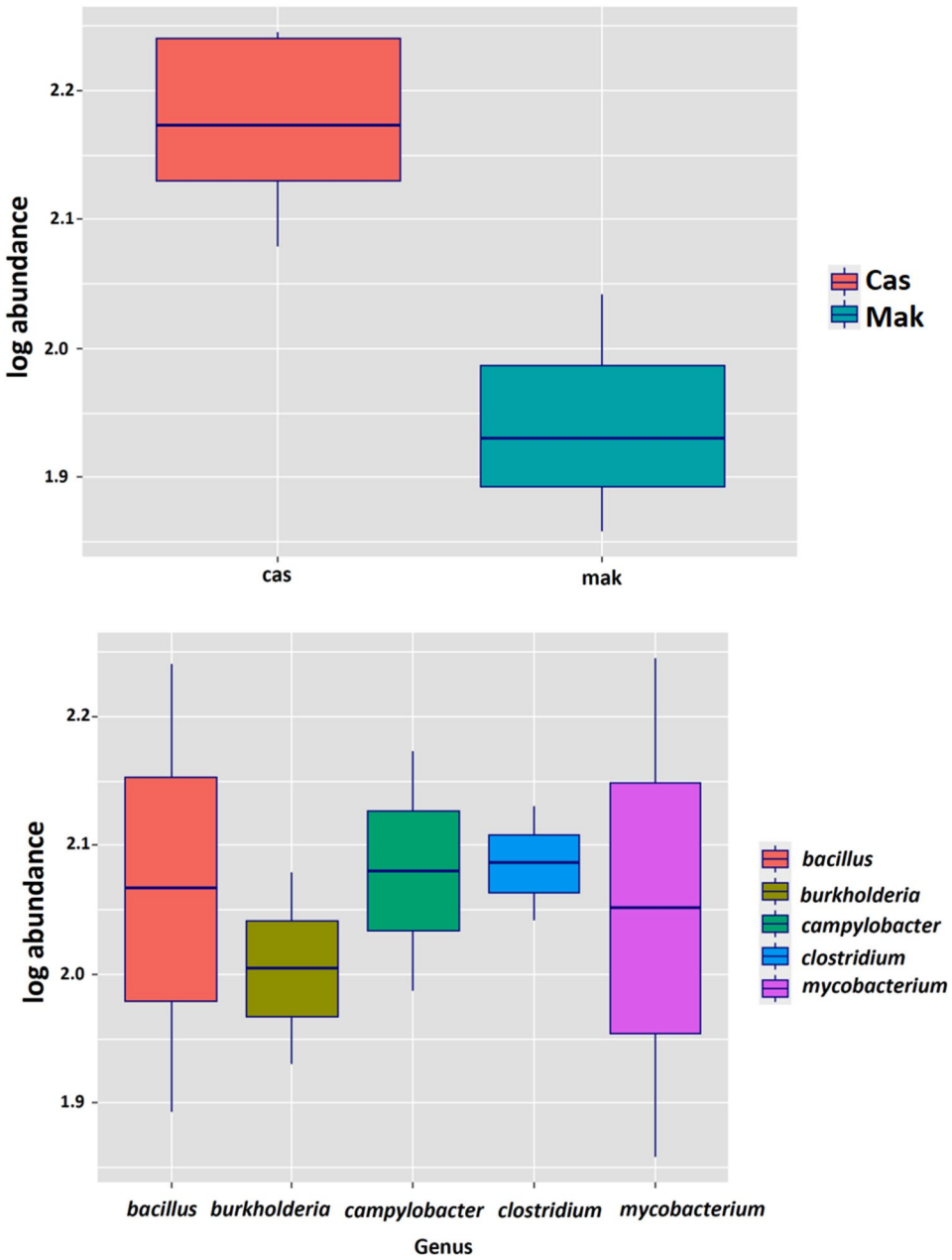

Fig. 3 Comparative pathogenic microbial abundance profile of Mak vs Cas (color figure online)

got (a) the overall soil physicochemical properties were alike as they belong to the same eco-geographical region and altitudinal level. (b) The overall bacterial diversity was more in Mak than Cas. (c) Moreover, Cas population contained more pathogenic genus than Mak. This clearly indicated a positive effect of organic manure in comparison to inorganic/chemical fertilisers. (d) The complementation values (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 is 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 is difficult to gain such knowledge at a lower taxonomic level (genus or species level).

Use of organic manure increased the microbial diversity of Mak

It has been documented previously that, continuous exposure of fertilization (both organic and inorganic) leads to the

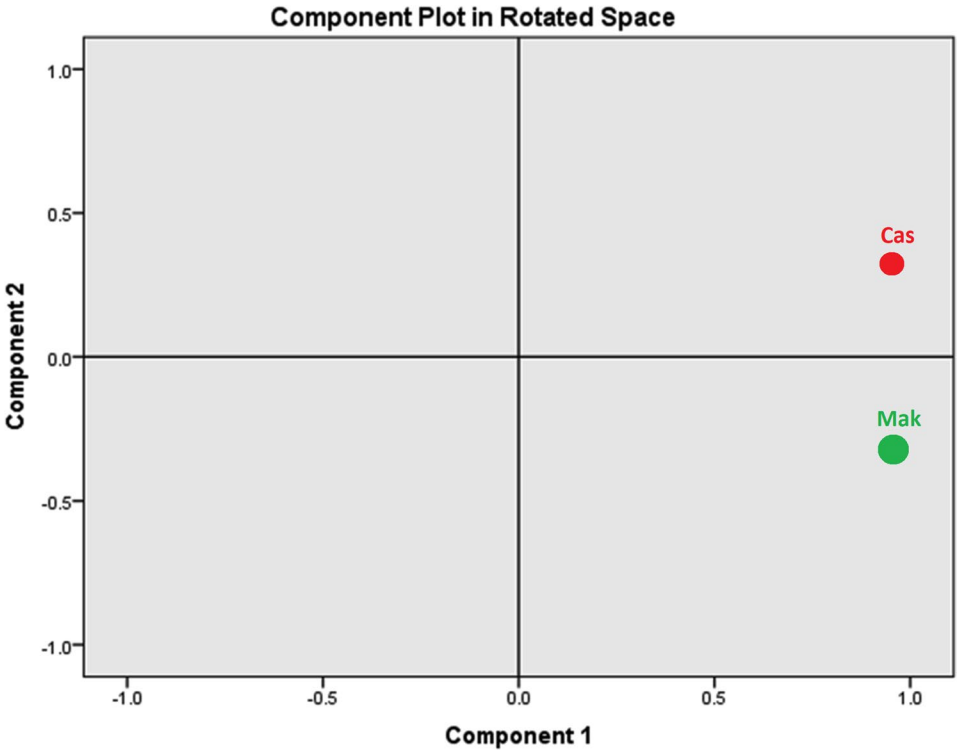

Fig. 4 PCA plot analysis of pathogenic microbial abundance profile of Mak vs Cas (color figure online)

addition of a specific category of carbon (C) and nitrogen (N) source to the soil. Over a period of time, 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 the 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 (Mak) tea garden is popular for using organic manure since its inception whereas, Castleton (Cas) uses inorganic fertilizers. These differential practices are thus, playing a major role in the microbial population between these two tea gardens.

Microbial populations of tea garden soil bear a relation with tea garden worker health

In the tea plantation sector, safety and security issues of workers are overlooked widely (Roy Chowdhury et al. 2014). Most of the workers are ignorant about the consequences of the exposure to chemicals, environmental factors, etc. 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; Rodríguez-Eugenio et al. 2018; Rajput et al. 2021; Bottone 2010; Ahmmed and Hossain 2016; http://www.tezu.ernet.in › project reports). Previous studies on tea garden workers showed the prevalence of neurological, gastrointestinal, renal and hepatic toxicity (Inglis and Sagripanti 2006; Frost 2001; Picard et al. 2005; Bae et al. 2002; Chenoll et al. 2015) among them. Most of the tea garden workers are prone to respiratory ailments such as tuberculosis and skin disorders (Gayathri and Arjunan 2019). 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 within their lactation period, and their children (Rajbangshi and Nambiar 2020). The difference in the soil micro-flora of Mak in comparison with Cas from our study indicated the role of fertilizers and chemicals in the development of soil microrganisms. The abundance of beneficial flora in Mak may provide a positive effect on the health aspect of tea garden workers further directed towards the advantages of organic manure over chemical fertilizers.

Conclusion

Human–microbe affiliation establishes even when the person is there in the mother's womb and the connection between human and soil microbes launches before it starts walking on the ground. It is believed that soil microbes contribute

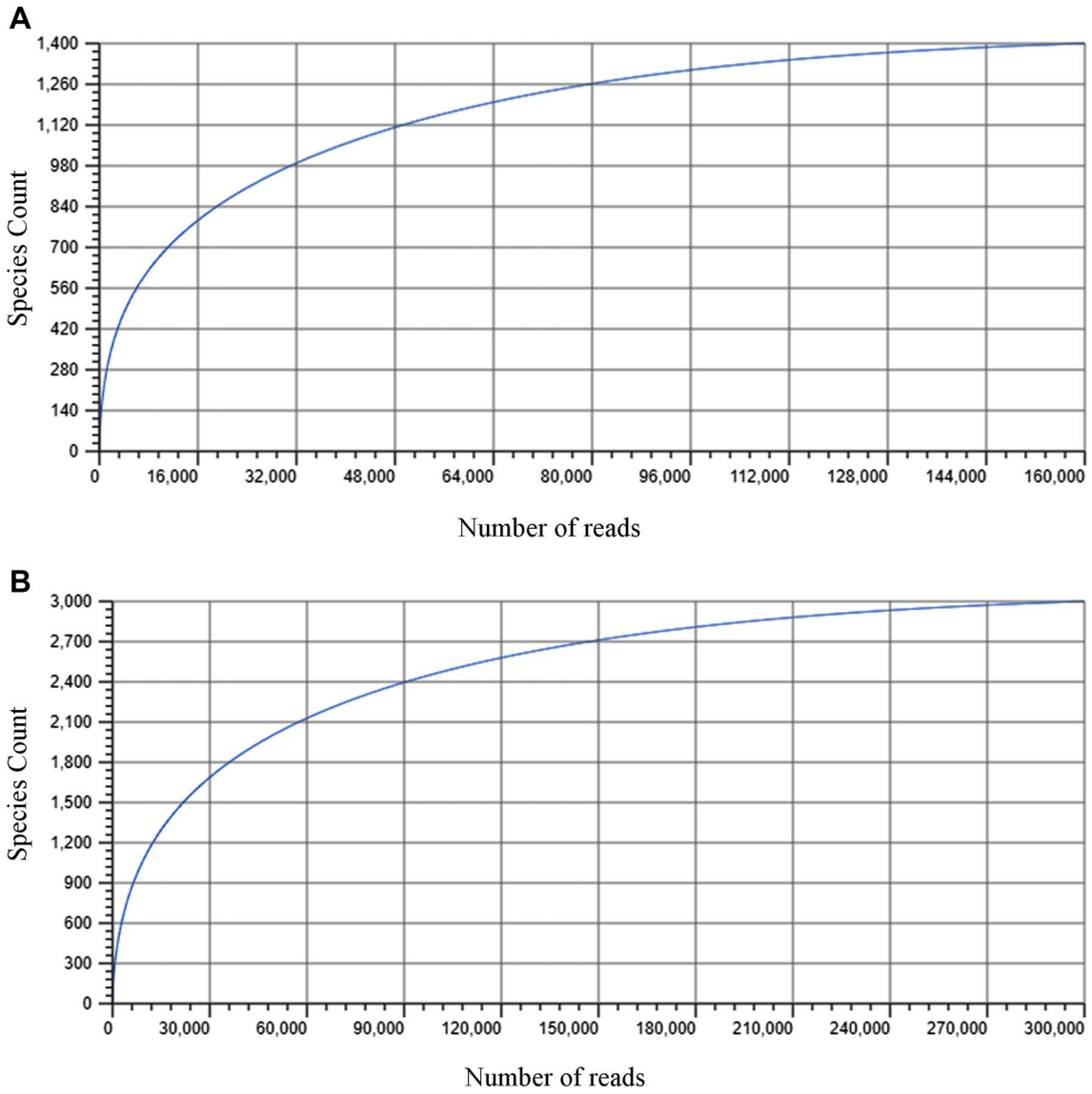

Fig. 5 Rare-fraction curve of (**a**) Mak and (**b**) Cas soil samples (color figure online)

considerably in developing the gut micro-flora and shape the overall human health. Soil dwellers, mainly soil micro-flora, play a paramount role in maintaining the biodiversity of a micro-habitat like tea gardens, paddy fields etc. The cultivable soil microbes are relatively easy to study while a large portion of culture-independent microbiomes remain largely illusory. In this consequence, soil metagenomics has become an important tool in studying the non-cultivable microorganisms present in a specific niche. In this present study, we did 16 s metagenomics of Makaibari (Mak) and Castleton (Cas) tea gardens from the Darjeeling region of India. The main difference between these two gardens is, Mak is an organic manure-based tea garden whereas Cas uses 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 overall 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 revealing volatility of the ecosystem. Finally, the number of human pathogens was more in Cas than Mak which supported the better tea garden worker health report in Mak over Cas. Literature survey, as well as our own survey also supports this fact. Thus, this study indicates that organic fertilizers have a positive effect on the soil microbial population in particular and human health in general in that region.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02635-6.

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Author contributions AS conceived the idea and did the experimental design. PK, GS, SC, MB collected and prepared samples for metagenomics. MB, PK and SB did the soil analysis related work. AS, GS and IS did the bioinformatics analysis. Figures and art works are mostly done by IS. All the authors contributed in manuscript writing and approved.

Declarations

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.

Research involving human and animal participants No animal or human were treated as sample in this study.

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