Original Research Article

Metagenomic Analysis of Bacterial Diversity on Tomato (*Solanum lycopersicum* L.) Leaves

V. Sumbula[1]*, P. Sainamole Kurian[1], D. Girija[1] and K. Anita Cherian[1]

[1]Department of Plant Pathology, [2]Department of Agricultural Microbiology, College of Horticulture, Kerala Agricultural University, Thrissur 680656, Kerala, India

*Corresponding author

A B S T R A C T

Plant leaves are colonized by complex microbial communities, which are adapted to the peculiar habitat on leaf lamina. Although the role and ecology of microorganisms in and on the leaves are only partially understood, leaf microbiota could have a beneficial role in plant growth and health. An amplicon metagenomic approach based on 16SrRNA gene on the MiSeq Illumina platform was used to identify the composition of bacterial communities associated with tomato leaves. A total of 44380 high quality 16s DNA sequences were clustered into 365 operational taxonomic units (OTUs). The results of metagenomomic analysis revealed that, tomato leaf bacterial community mainly comprised five dominant and four rare phyla, among which, *Firmicutes* (109 OTUs) was the most represented followed by *Proteobacteria* (95 OTUs), *Cyanobacteria* (46 OTUs), *Bacteriodetes* (43 OTUs) and *Actinobacteria* (16 OTUs). Rare phyla include, *Verrucomicrobia* (3 OTUs), *Acidobacteria* (2 OTUs) and *Gemmatimonadetes* (1 OTUs). Clostridia and Gammaproteobacteria was the most abundant classes of Firmicutes and Proteobacteria respectively. The dominant genera were *Geitlerinema* (33 OTUs), *Pantoea*(31 OTUs), *Clostridium* (26 OTUs) and *Acinetobacter* (24 OTUs). Besides the presence of common commensal leaf bacteria *viz. Pantoea Sphingomonas,* and *Acinetobacter,* the occurrence of *Clostridium*, a food, animal and human pathogenic genus and *Geitlerinema,* a fresh and marine water cyanobacteria on tomato leaves is an important observation of the present study. As a whole, this study describes the composition of the bacterial communities of tomato leaves, identifying a variety of genera that could exert multiple effects on growth and health of tomato plants.

Introduction

Microbial communities on or around plants play a major role in plant functioning and vigour. These microbial communities are mainly categorized into three types *viz.* rhizospheric, phyllospheric and endophytic microbial communities. Rhizospheric microbial communities, associated with root systems, have been extensively studied and best characterized, as they have been shown to be directly involved in crop productivity

through their roles in bio-accessibility of mineral nutrients, protection against pathogens and release of phytohormones to stimulate plant growth (Park *et al.,* 2008; Mendes *et al.,* 2013). The phyllosphere, which consists of the aerial parts of plants has been less intensively studied than the rhizosphere but has received considerable attention in recent years. Phyllosphere primarily of the set of photosynthetic leaves, is one of the most prevalent microbial habitats on earth. The leaf surface habitat is vast: vegetation modelling gives an estimated global leaf area of 508,630,100 km^2, which corresponds to 1,017,260,200 km^2 of upper and lower leaf surface, an area approximately twice as great as the land surface (Bulgarelli *et al.,* 2013).

The interest in phyllosphere microbiology was initially driven by investigations into plant pathogens, but most phyllosphere-colonizing microorganisms live as commensals and/or mutualistic symbionts on their host plants (Lindowand Brandl, 2003; Vorholt, 2012). Phyllosphere communities are involved in functional processes as large in scale as the carbon cycle, nitrogen fixation, and degradation of organic pollutants and pesticide residues (Müllerand Ruppel, 2014). They are also thought to be relevant for plant development and health as biofertilizers, phytostimulators and biopesticides to protect against invading pathogens. Furthermore, interactions in the phyllosphere zone determine the extent to which human pathogens are able to colonize and survive on plant tissues, an area of increasing importance with the rise in cases of human disease associated with consumption of fresh salad, fruit and vegetable produce (Whipps *et al.,* 2008). The third category is the endophytes; microorganisms which can colonize plants internally with- out any apparent adverse effects on the host (Gaiero *et al.,* 2013). There is a growing interest in endophytic microbes

on account of their potential use in plant growth promotion, antagonistic effect on pests and pathogens, alleviation of abiotic stress and in phytoremediation (Ryan *et al.,* 2008; Kurian, 2011; Mercado-Blanco and Lugtenberg, 2014). Being internal colonizers, they could provide a barrier against the invading pathogens directly or through the production of bio-active compounds.

The importance of bacterial diversity on and in the plants has an emerging field of research enabling to studying the various beneficial properties of phyllospheric and endophytic bacterial community. Even though plant leaves are ecologically and economically important ecosystem that hosts a large and diverse bacterial community, studies regarding microbial diversity on the leaves are limited. Thus, it is crucial to understand the community structure and diversity of phyllosphere and endophytic bacteria on plant leaves. It could further contribute to understanding the significant roles of plant microbiota in supporting their multiple bioactivities. Moreover, it will help to develop a more effective and less environmentally damaging strategies for improving plant health and plant protection. However, a vast majority of microorganisms are known to be non-amenable for cultivation on common media (Lundberg *et al.,* 2012; Thomasand Sekhar, 2014). Recently, the development of culture-independent high-throughput sequencing-based metagenomic analysis has further allowed us to obtain a global view about community structure and diversity of phylloplane and endophytic microbiome residing in plant tissues (Turner *et al.,* 2013). One of the main advantages of metagenomic approaches over culture-dependent methods, is the ability to theoretically detect all organisms that possess the targeted barcode gene. This includes, organisms that cannot or are extremely difficult to culture, which represent about

99% of the total estimated microbial diversity as well as rare taxa that are usually missed by culturing techniques (Price *et al.,* 2014). Culture-independent techniques can provide a more specific, replicable and detailed description of microbial diversity (Bulgari *et al.,* 2014).

Tomato (*Solanum lycopersicum* L.) is a vital solanaceous vegetable crop of global importance grown in an area of 4.8 million hectares (FAO, 2018). Varied climatic adaptability and high nutritive value made the tomato cultivation more popular in the recent years. This crop is well studied in terms of genetics, genomics, and breeding, thus being an excellent model for basic and applied research related to fruit quality, stress tolerance, and other physiologic traits (Panthee and Chen, 2010; Sahu *et al.,* 2012). Diseases are one of the main problems of the tomato industry all over the world, and the susceptibility of tomato to many pathogenic microorganisms leads to an intense use of agrochemicals (Gajanana *et al.,* 2006). Thus, biological control agents have emerged as an alternative approach for the control of tomato diseases. In this way, an increased knowledge of the ecology of bacterial communities associated to tomato plants will contribute to identify potential candidates for biologic control of tomato diseases and plant growth promotion. Therefore, the present study was aimed to assess the bacterial diversity of tomato leaves by metagenomic analysis.

Materials and Methods

Collection of samples

Tomato leaves were collected from research fields at College of Horticulture, Thrissur, Kerala (GPS coordinates: 10.5484 N 76.2857 E) by random sampling technique. Collected samples were kept in sterile plastic bags in a thermally insulated container. All samples were immediately placed on ice and transported to the high-tech seed testing laboratory attached to the Department of vegetable Science, College of Horticulture and stored at −80 °C prior to processing.

Metagenomic DNA extraction

The extraction of metagenomic DNA was done with an objective to construct metagenomic libraries of tomato leaf samples. In order to capture both endophytic and epiphytic fungi, the whole leaf tissue was used for DNA extraction. Collected samples were pooled and aseptically ground to a fine powder in liquid nitrogen using autoclaved pestle and mortar. Ground tissue powder aliquots (100 mg) were then stored at −80 °C. Metagenomics DNA extraction was done by using the DNeasy Plant Mini kit (QIAGEN, Dusseldorf, Germany). Samples were first mechanically disrupted and then chemically lysed. RNA is removed by RNAse digestion during lysis. Cell debris, precipitated proteins, and polysaccharides were removed and the sample was homogenized by centrifugation through a QIAshredder spin column. Buffering conditions were adjusted and the lysate was loaded onto the DNEasy Plant Mini spin column. During a brief spin, DNA selectively binds to the silica membrane while contaminants pass through. Remaining contaminants and enzyme inhibitors were removed in one or two efficient wash steps. The DNA was finally eluted using elution buffers to obtain 100 μl end product. The standard protocol provided in the user manual was followed to obtain the best result.

Qualitative and quantitative analysis of gDNA

Quality of gDNA was checked on 1% agarose gel (loaded 5 μl) for the presence of single intact band. The gel was run at 110V for 30 mins. 1 μl of each sample was loaded in

Nanodrop 8000 for determining A260/280 ratio. The DNA was quantified using Qubit dsDNA HS Assay kit (Life Technologies). 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

Preparation of libraries for 2 x 250 bp Run Chemistry

The amplicon library was prepared using Nextera XT Index Kit (Illumina inc.) as per the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). Primers for the amplification of the V3-V4 hyper-variable region (V3-Forward 5' CCTACGGGNBGCASCAG 3' and V4-Reverse 5' GACTACNVGGGTATC TAATCC 3') of 16S rDNA gene of bacteria were designed in Xcelris NGS Bioinformatics Lab. These primers were synthesized in XcelrisPrimeX facility.

The amplicon with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were purified by 1X AMpureXP beads, checked on Agilent DNA1000 chip on Bioanalyzer 2100 and quantified by Qubit Fluorometer 2.0 using Qubit dsDNA HS Assay kit (Life Technologies).

Cluster generation and sequencing

After obtaining the Qubit concentration for the library and the mean peak size from bioanalyser profile, library was loaded onto Illumina platform at appropriate concentration (10-20pM) for cluster generation and sequencing. Paired-end sequencing allowed the template fragments to be sequenced in both the forward and reverse directions on Illumina platform. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

Bioinformatics analysis and data processing

Sequence analysis was carried out using the quantitative insights into microbial ecology (QIIME) pipeline (Caporaso *et al.,* 2010). For sequence filtering, reads shorter than 200 bases or longer than 1,000 bases were discarded, sequences with homopolymer runs longer than six bases or more than six ambiguous bases were also discarded, whereas one barcode correction and two primer mismatches were accepted. Chimeras were removed using the UCHIME program[23]according to the USEARCH pipeline. Operational taxonomic units (OTU) were determined using the UCLUST algorithm (Edgar, 2010) at 97% sequence similarity. The taxonomy assignment of the bacterial OTU was carried out using the Naïve Bayesian RDP classifier with a minimum confidence of 0.8 (Wang *et al.,* 2007)against the Greengenes database downloaded from October 2012 (McDonald *et al.,* 2012). Singleton OTU was removed for statistical analysis. Demultiplexed raw sequence data were deposited in the Sequence Read Archive (http://www.ncbi.nlm. nih.gov/sra) under the accession number SRR10698108.

Results and Discussion

The libraries were prepared from the sample after amplifying V3-V4 region 16S segment. The 16S library mean size was 573bp. The library was sequenced using the Illumina 2 x 250 bp sequencing chemistry to generate ~150Mb of data per library. The next

generation sequencing was performed using 2x250 PE chemistry on the Illumina platform. Paired end sequence assembly was carried out for data generated using FLASH (Parameter Minimum overlap of 10 bases) assembler. FLASH (Fast Length Adjustment of Short reads) is a very fast and accurate software tool to merge paired-end reads from next-generation sequencing experiments. After the sequence assembly 458124 reads of bacteria were obtained and chimeric sequences were removed from these. Chimeras are DNA sequences composed of DNA from two or more microbial species. They are artefacts made during the PCR process. Hence, after filtering out these chimeric sequences, 443840 reads of bacteria were obtained. These good quality sequences from the samples were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity. OTUs are clusters of sequences, frequently intended to represent some degree of taxonomic relatedness, each resulting cluster is typically representing a same species. So, in the present study 1715 OTUs were picked up for bacteria and the total number of OTUs obtained after the removal of singletons was 709. As taxonomic assignment showed presence of large amount of chloroplast and mitochondrial contamination, they were removed from the assignment file before plotting the taxonomic charts and diversity calculation. So, the actual OTUs after the removal of contaminants was 365 (Table 1). Hence, after all the quality evaluations, a total of 443840 high quality sequences were recovered and assigned to 365 OTUs.

To confirm the accuracy of QIIME taxonomic assignments, rarefaction analysis was performed and it indicated that the sequencing depth had been saturated for the sample (Fig. 1). This curve is a plot of the number of species as a function of the number of sequences in a sample. The vertical axis displays the diversity of the community, while the horizontal axis displays the number of sequences considered in the diversity calculation.

Alpha diversity summarizes the diversity of organisms in a sample with a single number using different indices *viz.* Chao 1 estimator, Shannon index and Simpson's diversity index in a habitat/sample. In the present study Chao 1 estimator, Shannon index and Simpson's diversity index were 223, 4.12 and 0.97 respectively. These indices revealed that more than 50 per cent of the estimated bacterial richness was covered by the sequencing effort. The first definition of alpha diversity was proposed by Whittaker (1960) and is referred to as "The richness in species of a particular stand or community, or a given stratum or group of organisms in a stand". Alpha diversity is determined as the diversity within a single sample or set of replications. Chao 1 estimator index includes the observed number of species more specifically the number species singletons (species observed once) and doubletons (species observed twice). Shannon's index (H') measures for both abundance and evenness of the species present in sample. The Simpson index allows estimation the odds that two individual microorganisms sampled at random will belong to the same OTU.

Based on the metagenomic analysis of the complete 16S data set, tomato leaf bacteria were represented by total nine phyla include, five dominant phyla and four rare phyla (Fig. 2). Among different phyla identified, members of the phylum *Fermicutes* were predominant, collectively accounting 109 OTUs. It was followed by phylum *Proteobacteria* (95), unclassified (50), *Cyanobacteria* (46), *Bacteriodetes* (43) and *Actinobacteria* (16). Rare phyla include *Verrucomicrobia* (3), *Acidobacteria* (2) and *Gemmatimonadetes* (1). Predominance of *Fermicutes*, *Proteobacteria* and *Actinobacteria* as phyllosphere communities

of different agricultural crops (e.g. wheat, rice, apple, lettuce, and spinach) and naturally growing plants/trees had been reported by several researchers, although the proportions of individual taxa can vary depending on plant species and phenotype, geographical location, time of year, and human intervention (Redford *et al.,* 2010; Lopez-Velasco *et al.,* 2011; Knief *et al.,* 2012).

Population of *Fermicutes*in the sample is mainly grouped into four orders *viz. Bacillales*, *Lactobacillales*, *Clostridiales* and *Selenomonadales* (Table 2). Among these, members of *Clostridiales* were predominant sharing 82 OTUs, and followed by those of Bacillales (7 OTUs), *Selenomonadales* (7 OTUs) and *Lactobacillales* (2 OTUs). Order *Clostridiales* majorly composed of the genera*Clostridium* (26 OTUs), *Eubacterium* (15 OTUs), *Faecalibacterium* (15 OTUs), *Blautia* (11 OTUs), *Ruminococcus* (10 OTUs) and *Roseburia* (5 OTUs). The well documented habitat of genus *Clostridium* is soil, water and decaying organic matter and most of them are capable to survive as saprophytes. But recent studies reveal plants as hosts for epi- or endophytic colonization of *Clostridium* suggesting possibility of plants as alternate host for major human and animal bacterial pathogens (Kirzinger *et al.,* 2011; Timmers *et al.,* 2012; Zeiller *et al.,* 2015). Pyrosequencing of grapevine leaf microbial communities shows that, most OTUs from grapevine leaves were assigned to genera *Clostridium* and *Staphylococcus* (Yousaf *et al.,* 2014). Similarly, Ercolani (2017) reported occurrence and persistence of culturable *Clostridium* spp. on the leaves of horticultural plants such as basil, celery, lettuce and tomato. In the present study, most abundant class in the phylum *Proteobacteria* was *Gammaproteobacteria* (65 OTUs) followed by *Alphaproteobacteria* (15 OTUs),

Betaproteobacteria (3 OTUs) and *Deltaproteobacteria* (2 OTUs) (Fig. 2). The genera *Pantoea*, *Acinetobacter* and *Sphingomonas* were predominant in phylum *Proteobacteria.* The phylum *Proteobacteria* comprises several species that promote plant growth and also act as biologic control agents of different diseases. A study of bacteria associated to tomato leaves revealed the presence of Proteobacteria both in greenhouse and field-grown plants (Enya *et al.,* 2007) and also identified *Bacillus* (phylum *Firmicutes*) and *Pantoea* (phylum *Proteobacteria)* strains with strong antifungal activity against tomato pathogens such as *Botrytis cinerea* and *Alternaria solani*. Some *Pantoea* species can produce *N*-acyl-homoserine lactone (AHL) and the plant-growth hormone indole-3-acetic acid (IAA)(Enya *et al.,* 2007), fix nitrogen from the atmosphere (Loiret *et al.,* 2004) and establish quorum sensing systems on leaves, which makes them possible to suppress pathogens on leaves (Frances *et al.,* 2006; Pusey *et al.,* 2011). *Sphingomonas* spp. is a Gram-negative, rod-shaped aerobic bacterium that is a highly competitive plant leaf colonizer. In a series of experiments, researchers demonstrated that the leaf colonising bacterium *Sphingomonas* spp. could protect plants against the leaf-pathogenic *Pseudomonas syringae* through substrate competition (Innerebner *et al.,* 2011). Carbon partitioning plays an important role for *Sphingomonas* spp. to be effective antagonists in the phyllosphere (Delmotte *et al.,* 2009). Similarly, some strains of *Acinetobacter* play an important role in plant growth promotion, as certain strains of this genus are known to be involved in phytostimulation based on the production of plant-growth-promoting hormones, solubilization of phosphate and production of siderophores (Sarode *et al.,* 2009; Sachdev *et al.,* 2010) (Fig. 3).

Table.1 Reads statistics of 16S metagenome data

PE Reads	Total Reads (R1+R2)	Flash Reads	Chimeric Sequences	Pre-processed Reads (Non-chimeric sequences)	Total OTUs	Total OTUs After Singleton Removal	Total OTUs after contamination removal
464519	929038	458124	14284	443840	1715	709	365

Table.2 Genus-level taxonomic assemblage of bacterial diversity from predominant phyla

Phylum	Class	Order	Family	Genus
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium(3)
		Coriobacteriales	Coriobacteriaceae	Collinsella (8)
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides (21)
			Prevotellaceae	Prevotella (8)
			Rikenellaceae	Alistipes (6)
Cyanobacteria	unclassified (derived from Cyanobacteria)	Chroococcales	Unclassified	Cyanothece (2)
		Nostocales	Nostocaceae	Aphanizomenon (3)
		Oscillatoriales	Unclassified	Geitlerinema (33)
Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus (5)
			Paenibacillaceae	Brevibacillus (2)
		Lactobacillales	Lactobacillaceae	Lactobacillus (2)
	Clostridia	Clostridiales	Clostridiaceae	Clostridium (26)
			Eubacteriaceae	Eubacterium (15)
			Lachnospiraceae	Roseburia (5)
			Ruminococcaceae	Faecalibacterium (15)
				Ruminococcus(10)
			Unclassified	Blautia (11)
	Negativicutes	Selenomonadales	Acidaminococcaceae	Acidaminococcus(4)
			Veillonellaceae	Dialister(3)
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas (15)
	Betaproteobacteria	Unclassified	Unclassified	Unclassified (3)
	Deltaproteobacteria	Unclassified	Unclassified	Unclassified (2)
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea(31)
		Pseudomonadales	Moraxellaceae	Acinetobacter(24)
		Vibrionales	Vibrionaceae	Vibrio (5)
		Xanthomonadales	Xanthomonadaceae	Stenotrophomonas (3)
		unclassified (derived from Gammaproteobacteria)	unclassified (derived from Gammaproteobacteria)	unclassified (derived from Gammaproteobacteria) (2)
Unclassified (derived from Bacteria)	Unclassified	Unclassified	Unclassified	Unclassified (50)

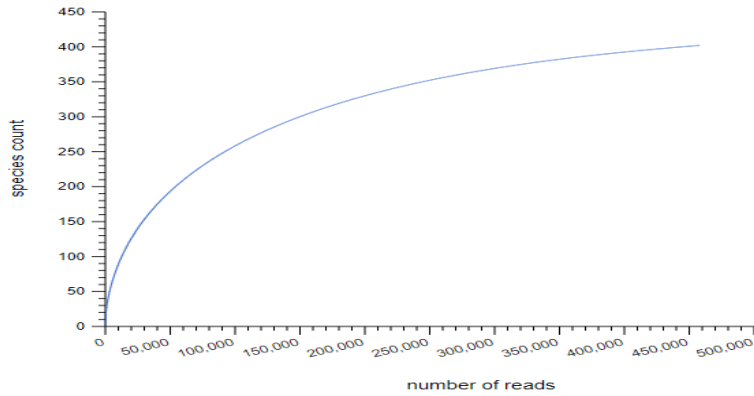**Fig.1** Rarefaction curve for bacterial OTUs, clustering at 97% sequence similarity

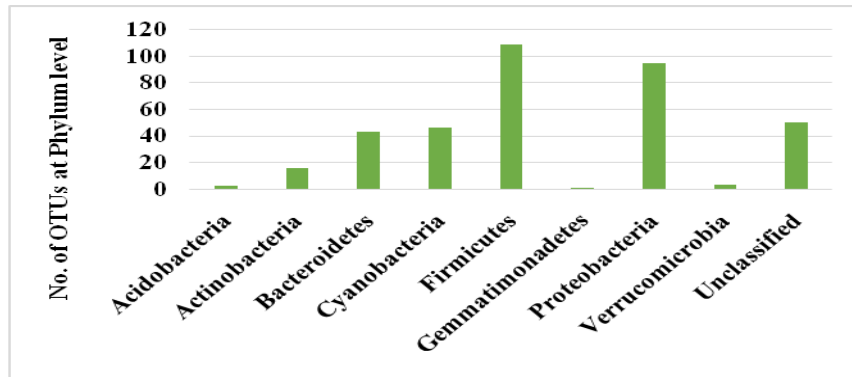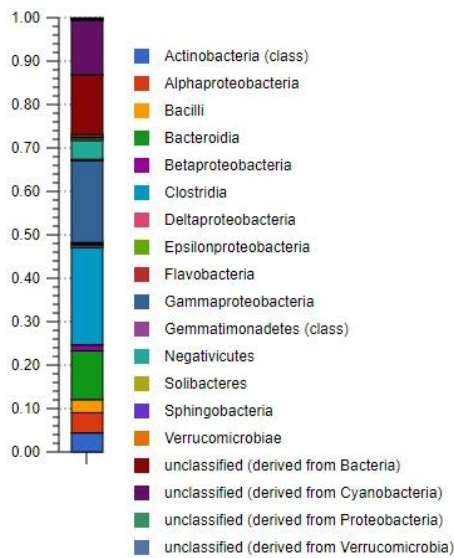**Fig.2** Operational taxonomic units of different phyla

Fig.3 Bacterial classification using RDP Classifier at 97% identity shown at class level

Members of order oscillatoriales were found to be abundant under phylum *Cyanobacteria* with *Geitlerinema* as abundant genus. To the best of the authors' knowledge this is the first report of presence of *Geitlerinema*on tomato leaves. Genus *Geitlerinema* is filamentous cyanobacteria commonly inhabiting fresh and marine water. However, different studies have shown increased occurrence of these bacteria in recent decades due to climate change. The filaments and other propagules are being transferred over long distances by air current and wind leading to distribution of these organisms from their source area to other (Broady, 2005 and Pearce *et al.,* 2009). There are reports showing the presence of *Geitlerinema*in air samples (Genitsaris *et al.,* 2011) and as epiphytes on rice (Zeinab *et al.,* 2013)and mangrove (Alvarenga *et al.,* 2015). Table 1 shows that, the genus *Bacteroides* and *Collinsella* dominate among the phyla *Bacteroidetes* and *Actinobacteria* respectively. Bacteria belonging to *Acidobacteria*, *Gemmatimonadetes* and *Verrucomicrobia* were less abundant and less diverse on tomato leaves.

In conclusion, the results of the present study indicate that, leaves of tomato plants support a large diversity of bacteria with an abundance of bacterial population from phyla *Fermicutes* and *Proteobacteria*. The dominant bacterial genera on the tomato leaves were *Geitlerinema* (33 OTUs), *Pantoea* (31 OTUs), *Clostridium* (26 OTUs) and *Acinetobacter* (24 OTUs). The presence of commensal bacterial genera *viz. Pantoea, Sphingomonas, Acinetobacter* are considered as beneficial to the plant, since they have important role in pathogen exclusion, plant growth and productivity. Even though, presence of *Clostridial* species on tomato leaves was unexpected outcome of present study it certainly merits greater attention because its spores can be added to the tomato fruits leading to contamination of food. Occurrence

Geitlerinema on tomato leaves is another important observation of present study. Moreover, it is important to note that, the unclassified taxa were found to be abundant in the sample and this might indicate that the majority of the leaf bacteria are undiscovered and the activity of these bacteria is still unknown. Regardless of the ratio, the composition of bacterial taxa as whole might play a huge role in mediating the health of tomato plants.

Acknowledgement

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