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ORIGINAL PAPER

The endosphere bacteriome of diseased and healthy tomato plants

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Abstract
Here we analyze the microbial community of healthy and diseased tomato plants to evaluate its impact on plant health. The organisms found in all samples mainly belonged to 4 phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. The *Proteobacteria* were the highest relative abundant within the endophytic communities of different plant organs of diseased tomato. Among endophytic bacteria of tomato, only a few taxa could be cultured. Here we showed that only a few taxa of bacteria inhabiting tomato plants could be cultured and that all plant organs have a highly diverse endophytic bacterial, whose activity might affect plant growth and development as well as health. The roots seem to be an important barrier for microbes and leaves appear to be the organs with the higher diversity which is incidentally related to plant health. Fruits also contain a complex bacterial community that appeared to be unaffected by foliar diseases such as gray leaf spot at least under the conditions studied.

Keywords Endophytes · Tomato · Metagenomic · Bacterial biocontrol · *Stemphylium lycopersici*

Introduction

The plant microbiome is formed by the genomes of microorganisms living in association with plants, which led to new ideas in terms of plants evolution. This is because selective forces act not only on the plant genome but also exert selective pressure on the associated microbes (Hardoim et al. 2015). The diversity and size of these communities form a complex array that is known as the second genome of a plant (Berendsen et al. 2012). So, the microbiome of a plant consists of a mixture of mutualistic, commensals as well as putative pathogenic communities of microorganisms (Hardoim et al. 2015; Frank et al. 2017). Plants are large and have diverse niches that might host an ample array of microorganisms, among them bacteria, known as bacterial endophytes (Azevedo et al. 2000). They are adapted to live within intercellular spaces and might contribute in different ways to plant growth and/or resistance against biotic stress factors by producing bioactive molecules (Berg et al. 2017). The ubiquitous presence of saprophytic as well as pathogenic microorganisms in plants raises the question whether the outbreak of diseases is the result of an environmental stress on plant´s microbiome, within the whole plant or just the affected organ and/or tissue. High-throughput sequencing technologies such as Illumina are key tools to study microbial communities of plants (Yan et al. 2017).

Tomato (*Solanum lycopersicum* L.) is among the most important vegetables considering production as well as consumption around the world. It is affected by several diseases provoked by fungi, bacteria and viruses (López et al. 2018). *Fusarium* spp., *Stemphylium* spp., *Verticillium dahliae*, *Sclerotinia sclerotiorum*, *Phytophthora infestans*, *Rhizoctonia solani* and *Pyrenochaeta lycopersici*; *Clavibacter michiganensis* ssp. *michiganensis*, *Ralstonia solanacearum*, *Tomato yellow leaf*

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curl virus, *Tomato chlorotic dwarf viroid*, *Tomato mosaic virus*, and *Tomato streak virus* are among the most important pathogens of tomato (Farr and Rossman 2016; Kil et al. 2016; Lowe-Power 2017; Franco et al. 2017a, b; Raimondo and Carlucci 2018; Nandi et al. 2018). Gray leaf spot is an endemic fungal disease in most tomato producing areas; it is caused by three species of the genus *Stemphylium* (Franco et al. 2017a, b). Symptoms are small brown spots that turn into slightly angular gray lesions surrounded by a yellow halo. In severe attacks, spots within the entire leaf merge into large necrotic follicular areas (Blancard 2012; Jones et al. 2014). The disease is controlled using synthetic fungicides, compounds that might be harmful to the environment as well as human health (Ippolito and Nigro 2000). In addition to this, fungi develop resistance in a short period of time (Rosslenbroich and Stuebler 2000); therefore, there is a need to develop alternative strategies of disease management, like the use of microbes as biological control agents (Kefi et al. 2015).

Considering foliar diseases, the microbial communities that are most probably affected are those occupying intercellular spaces within the leaf tissue. The comparison of soil bacterial communities, where diseased or healthy plants developed, showed that specific bacteria are associated with pathogen suppression (Li et al. 2014; Lee et al. 2017). Comparisons of soil samples microbial communities with or without soilborne pathogens paved the way to understand the interactions within microbial communities, which led to the identification of microorganisms that control or compete with pathogens. Kwak et al. (2018) found that disease-resistant tomatoes recruit bacterial allies in their rhizospheric microbiome, to protect themselves from infection. This differs from those studies using disease-suppressive soil, in which plants are disease susceptible but where severity is reduced as a result of changes in the soil microbiome in response to pathogen build-up, where specific microbial taxa are enriched within the resistant plant. Therefore, to manage pathogens and the diseases they provoke it is particularly important to study microbial communities (Lee et al. 2017; Kwak et al. 2018).

Vegetables grown in greenhouses are threatened by more diseases than in the field (Abawi and Widmer 2000; Li et al. 2014), mainly due to the prevailing conditions regarding temperature, humidity, salinity, and tillage, what frequently enhance pathogens population growth (Li et al. 2014). The purpose of this study was to analyze the microbial community composition of healthy and diseased tomato plants to evaluate how changes in the microbiota impact plant health.

Materials and methods

Site and sample collection

Tomato plants (cv. Elpida) were grown in a greenhouse close to the city of La Plata, Buenos Aires province, Argentina. Several plants presented necrotic symptoms that look like tomato gray leaf spot and were associated with *S. lycopersici*.

Plant tissues samples (roots, shoots, leaves and fruits with and without symptoms) from three diseased and three healthy plants were collected separately at the final flowering stage in May 2017.

Roots of diseased and healthy of three tomato plants were cut off and were gently shaken to remove loosely adhered soil. Each sample was stored at 4 °C until it was brought to the lab for immediate processing. Genomic DNA of healthy and diseased samples was used as templates for high-throughput sequencing. Samples also were used for the isolation and identification of bacterial species.

Identification of *S. lycopersici* in tomato samples with diseases symptoms

Fungal isolates were obtained from tomato plants with typical symptoms of gray leaf spot and were analyzed regarding morphological characteristics both in cultures grown on homemade and commercial potato dextrose agar (PDA) as described by Franco et al. (2017a).

Total genomic DNA was extracted from axenic cultures using the CTAB method of Bornet and Branchard (2001), whose quality and quantity was evaluated by electrophoresis in a 0.7% agarose gel. Genomic DNA was quantified by comparing the DNA bands with those of a molecular marker with the Gene Tools image analyzer (SynGene, Cambridge, UK). Extracted DNA was stored at − 80 °C until analysis.

The ITS1 and ITS2 regions of *S. lycopersici* were amplified using primers ITS4 and ITS5 (White et al. 1990; Franco et al. 2017a). Primers GPD (forward and reverse), which were designed based on *gpd* sequences of *Stemphylium* spp. available in the GenBank (www.ncbi.nlm.nih.gov), were used to amplify a partial sequence of the *gpd* gene (Franco et al. 2017a). PCR products were purified and sequenced at Macrogen Inc. (Seoul, Korea). The ITS1-ITS2 and *gpd*, partial sequences were deposited in the DDBJ/EMBL/GenBank under accession numbers MK905413–MK905416 and MK908104–MK908106, respectively.

DNA isolation and amplicon sequencing from plant tissues

DNA was isolated from leaves, shoots, roots and fruits samples of three healthy and three diseased tomato plants.

Plant tissues were surface disinfected with 5% commercial bleach and 0.01% Tween 20 for 5 min and rinsed with sterile distilled water. To check the efficiency of samples sterilization, the water of the last wash was plated on TSA (TSA - Britania) and also aliquots were included in PCRs aimed at amplifying the *16S rRNA* gene (López et al. 2018). Samples of each tissue were homogenized in 0.95% (w/v) NaCl, filtered through 0.45 µm organic filter membranes (©GVS) and centrifuged (10 min; 15,000×*g*) to separate plant debris from bacteria. Pelleted bacteria were used to extract genomic DNA with the Wizard® Genomic DNA purification Kit (Promega) following a previously described protocol (Romero et al. 2014; López et al. 2018). The DNA obtained was dissolved in 50 µl of rehydration solution, quantified by electrophoresis and stored at − 20 °C for further analysis.

The *16S rRNA* gene V1–V3 region was amplified using 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) primers, with a barcode on the forward primer for MiSeq instrument (Illumina Inc., San Diego, CA). PCR was performed as described by López et al. (2018). Sequencing was carried out at MR DNA (http://www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequences were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) and are available under accession number PRJNA438294.

The demultiplexed and Phi-X174-free reads were overlapped with fastq-join v.1.3.1 with a minimum overlap of 40 bp and a maximum of 15% difference in the overlapping region. The overlapped reads of the prokaryotic (Bacteria and Archaea) library were initially classified with an 80% bootstrap cutoff to the Ribosomal Database Project (RDP-II) *16S rRNA* reference database, training set v.16 MOTHUR-formatted (https://mothur.org/wiki/RDP_reference_files), with MOTHUR v.1.40.0 (https://www.mothur.org/). This initial step removed reads belonging to mitochondria, chloroplast and unidentified sequences at kingdom level (unknown). Then, using the software SEED2 v.2.1.05 (http://www.biomed.cas.cz/mbu/lbwrf/seed/) prokaryotic sequences were trimmed and clustered. Initially, specific primers were trimmed and then ambiguous and sequences shorter than 300 bp as well as reads with an average read quality lower than Q30 were discarded. Secondly, chimeric reads were removed by VSEARCH "De Novo" v.2.4.3 (Rognes et al. 2016) implemented in SEED2 and OTUs were clustered with the same tool at 97% similarity. Finally, the OTU table was saved and OTUs accounting for less than 0.005% of the sequences were removed according to Bokulich et al. (2013) for further analyses. The most abundant OTU sequences were retrieved in SEED2 and classified as described. This classification was considered as the taxonomic information of each OTU up to genus level when possible.

The graphic representation of the community structure at the phylum level of all the samples studied, tissues of healthy and diseased plants, Fig. 2 was done with the open-source software Circos (Krzywinski et al. 2009). A more detailed description at the genus level analyzing roots and leaves samples was represented with bar charts in Figs. 3 and 4.

Isolation of bacteria from tomato tissues

Endophytic bacteria were isolated from leaves, shoots, roots and fruits samples of healthy as well as diseased tomatoes (Elpida F1, Enza Zaden). Samples were surface sterilized as described above. Sterile tissues were crushed and homogenized in 3 ml of 3 X Ringers solution and aliquots of 100 µl of the supernatants were plated on the three media (TSA, King B and nutritive agar - Britania), plates were incubated at 28 °C for 5 days (Surette et al. 2003; López et al. 2018). After a 5 day-incubation period, colonies are developed. They were sub-cultured until pure cultures were obtained and then, they were morphologically characterized in terms of size, shape and color and were. Isolated bacteria were grown in liquid media until saturation and aliquots were mixed to make a final concentration of 10% glycerol, and were stored at − 80 °C.

Isolation of genomic DNA, PCR amplification and sequencing of 16S rRNA gene

Bacterial DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega) from aliquots of 1×10^9 cells.ml^{-1} liquid cultures. The quality and quantity of the isolated DNA was checked by electrophoresis.

The organism identities were initially analyzed using the 1.5 kb sequence coding for the *16S rRNA*. Such fragments were amplified by PCR in a thermocycler (MinicyclerTM–MJ Research), employing primers 27f and 1492r (Weisburg et al. 1991; López et al. 2018). PCR products were purified and sequenced. The *16S rRNA* gene sequences determined in this study have been deposited in the GenBank database under accession numbers MH915620–MH915655.

Sequence analysis and alignment were performed using Geneious R9 software (Geneious version R9.0, Biomatters, http://www.geneious.com, Kearse et al. 2012). Sequences were aligned with MEGA 5.10 (Tamura et al. 2011) using the default parameters of the ClustalW and the alignments were visually checked and manually optimized. Phylogenetic analysis was performed under Maximum-likelihood criteria. Clade stability was assessed via 1000 bootstrap replications using the heuristic search options described above.

Results

Identification of *S. lycopersici* on diseased tomatoes

Based on cultural and morphological features as well as ITS1-ITS2 and *gpd* sequences, the pathogens collected from tomato plants with typical symptoms of tomato gray leaf spot were identified as *S. lycopersici* (Fig. 1).

Bacterial communities within tissues of healthy tomato plants

The endophytic bacterial community of tomato plants was identified by high-throughput sequencing on the MiSeq platform; we analyzed two replicates of roots, shoots, leaves and fruits of healthy and diseased tomato samples. The analysis provided up to 1,163,222 sequences that ended up in 137,472 high–quality sequences, once low-quality reads were removed (13% of initial sequences) (Table 1). Approximately 80 and 73% high-quality *16S rRNA* gene sequences obtained from healthy and diseased samples, respectively, were annotated as chloroplast and were filtered to remove plant-derived OTUs.

Finally, a total of 60,055 reads from healthy and 77,417 reads from symptomatic tissues were obtained (Table 1). They were distributed within 218 OTUs, at a 97% identity. OTUs comprised 116 bacterial taxa that were clustered at the genus level, among them, 108 were shared by healthy and diseased tomatoes. The Good´s coverage indicated that the depth of sampling in the sequencing process was higher than 82% for trimmed and normalized data (Table 1), which suggests that most representatives of the original communities are represented in the analysis.

Fig. 1 Tomato leaf with characteristic symptoms of gray leaf spot consisting of small brown spots

Table 1 The Good's coverage, initial and final reads of healthy and diseased samples

Sample	Initial reads	Final reads	Good's coverage (%)
Healthy roots	86,066	1658	92.42
	80,870	535	89.53
Symptomatic roots	69,452	2691	97.32
	73,880	689	91.15
Healthy shoots	49,082	525	94.10
	94,275	11,015	99.12
Symptomatic shoots	25,004	219	88.13
	89,009	13,302	99.11
Healthy fruits	47,199	2649	97.09
	82,789	42,852	99.75
Symptomatic fruits	55,452	2361	96.57
	80,818	42,629	99.68
Healthy leaves	92,602	561	88.77
	55,616	260	82.69
Symptomatic leaves	86,209	12,676	99.29
	94,899	2850	96.84

The alpha diversity of tomato endophytes was calculated by using Hill numbers (Hill 1973), ^{0}H (richness), ^{1}H (diversity) and ^{2}H (evenness). Roots and shoots of diseased tomatoes have a richer (^{0}H) and more diverse (^{1}H) bacterial community than those of healthy plants (Table 2). On the contrary, the endophytic community of leaves of healthy plants is richer and more diverse than that of diseased plants. In addition to this, roots and shoots of diseased plants as well as leaves from healthy plants have a greater evenness (^{2}H) of species. Regarding fruit samples, the Hill indexes suggested that they present similar endophytic communities (Table 2).

Microbial communities within tissues of different organs of healthy plants showed that the community of leaves is

Table 2 Indexes of richness, diversity and evenness within the organs of healthy and diseased plants

Sample	Richness, diversity and equitability estimator		
	^{0}H	^{1}H	^{2}H
Healthy roots	25.50 ± 2.05	5.99 ± 0.63	2.53 ± 0.19
Symptomatic roots	36.00 ± 0.71	13.67 + 0.18	6.86 ± 0.15
Healthy shoots	22.00 ± 0.57	4.91 ± 0.24	2.55 ± 0.10
Symptomatic shoots	31.00 ± 0.71	7.23 ± 0.38	3.02 ± 0.12
Healthy fruits	33.50 ± 1.48	7.67 ± 0.34	3.06 + 0.09
Symptomatic fruits	33.50 ± 1.63	8.87 ± 0.65	3.93 ± 0.25
Healthy leaves	41.50 ± 0.35	16.00 ± 0.17	8.40 ± 0.12
Symptomatic leaves	25.50 ± 1.91	9.48 ± 1.10	5.71 ± 0.63

the richest (^{0}H), the most diverse (^{1}H) and the one with the highest evenness (^{2}H), compared to communities of other organs. The endosphere bacteriome of diseased plants had different indexes along with all tissues, but leaves were particularly affected, which is reflected by a decrease of the three indexes that incidentally increased in roots, shoots and fruits (Table 2).

Composition of endophytic communities within organs of diseased and healthy plants

The organisms found in all samples mainly belonged to 4 phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, being the first and the latter ones the most abundant ones (Fig. 2).

When the composition of the endophytic communities within different plant organs of diseased plants was analyzed, it was found that in roots, shoots and fruits, the highest relative abundance corresponded to the phylum *Proteobacteria* (31.71%, 50.76% and 62.53%, respectively). In shoots and fruits, the second most abundant phylum corresponded to *Actinobacteria* (6.1% and 25.21%, respectively) and in roots *Firmicutes* (18.73%). Healthy and symptomatic shoots presented a higher relative abundance of *Shinella*, *Acidovorax,* and unclassified *Rhizobiales*, and in the samples of fruits of healthy and diseased plants, the most abundant genera were *Pseudomonas*, *Curtobacterium,* and *Pectobacterium*.

Symptomatic leaves presented a greater relative abundance of *Actinobacteria*, which was higher than in any other sample analyzed. If we compare the communities within leaves of diseased and healthy plants, a substantial increase in the abundance of *Actinobacteria* occurred. In symptomatic leaves, where *Actinobacteria* represented 58.28% and was accompanied by a decrease in *Proteobacteria* from 32.86 to 25.72%. In contrast, the endosphere bacteriome of healthy leaves presented a 15.73% of *Actinobacteria*. Whether the plant was diseased or not the relative abundance of *Firmicutes* remained the same (Fig. 2).

Regarding the endosphere bacteriome of shoots and fruits of *Stemphylium* diseased plants, their composition was like that of healthy shoots and fruits these organs are affected once the disease has evolved considerably (Fig. 2).

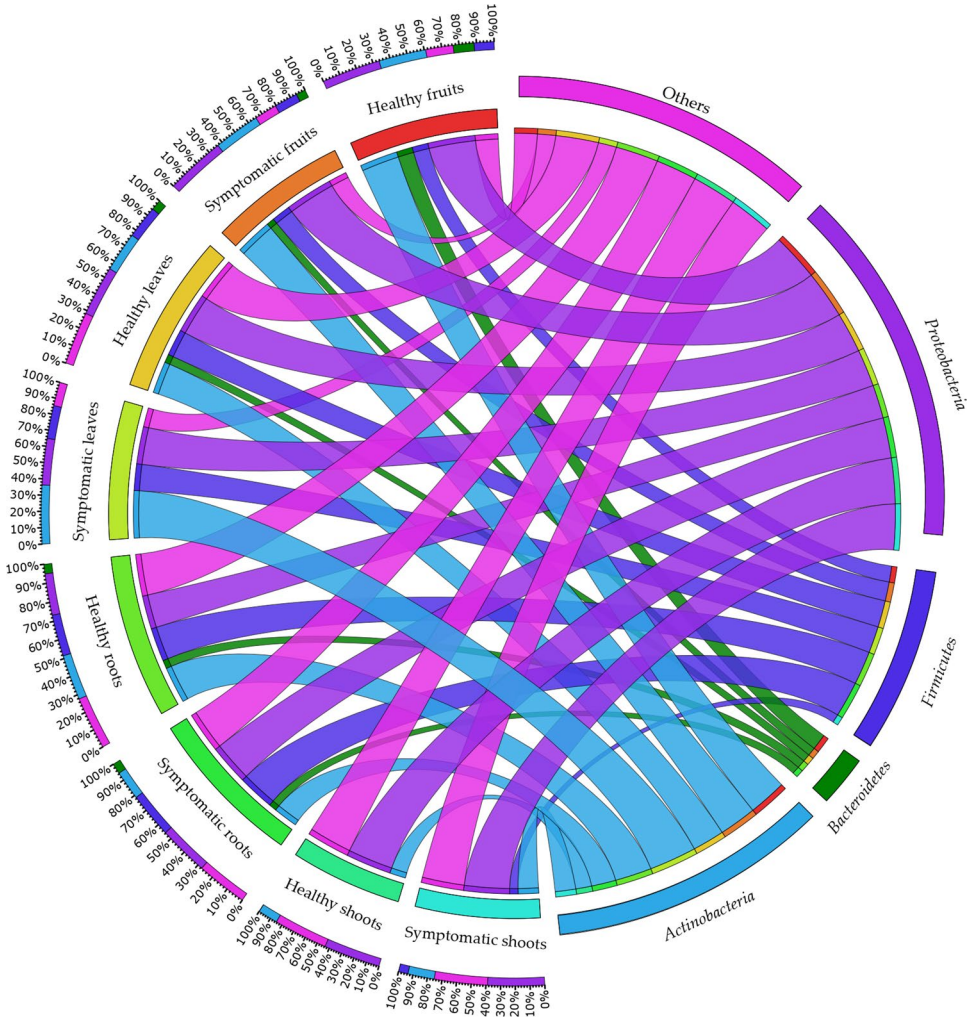

Fig. 2 Bacterial community structure at phylum level in roots, shoots, fruits and leaves of healthy and symptomatic tomato plants (Krzywinski et al. 2009). Phyla with a relative abundance < 0.5% and unclassified bacteria were grouped in "Others"

A characteristic feature of roots was the greater abundance of *Firmicutes* compared to other organs. Healthy plants roots presented a higher relative abundance of *Actinobacteria* compared to those of diseased plants 23.30% and 7.4%, respectively. Additionally, the roots of healthy plants presented a lower abundance of *Proteobacteria*, mainly within the *Gammaproteobacteria* class that was reduced from 31.70 to 15.0% (Fig. 2).

An analysis of diversity and abundance at the class level in healthy and diseased roots (Fig. 3) showed that while in former ones the most abundant class was *Actinobacteria* (23.30%) followed by *Bacilli* (15.02%) and *Gammaproteobacteria* (12.58%); in the latter ones was *Gammaproteobacteria* (28.20%) followed by *Bacilli* (17.58%). In the roots of diseased plants, *Actinobacteria* were reduced to only 7.43% (Fig. 3). In both samples, more than 40% of the microorganisms were unclassified bacteria and were grouped with the classes whose relative abundance was less than 0.50% in "Others".

Interestingly, *Curtobacterium*, *Staphylococcus* and *Clavibacter* were the most abundant genera in healthy roots (11.86%, 10.36% and 5.41%, respectively), while *Pectobacterium*, *Staphylococcus* and *Pseudomonas* were those with major relative abundance in symptomatic samples (16.09%, 11.01% and 6.22%, respectively).

Healthy leaves mainly contained *Gammaproteobacteria* and *Actinobacteria* that represented 20% and 15%, respectively (Fig. 4) and were accompanied by a large proportion of unclassified bacteria 43%. Healthy leaves contained mostly *Pseudomonas* (16.5%), *Propionibacterium* (9.5%) and *Streptococcus* (7%) (Fig. 4). On the contrary, *Actinobacteria* was by far the main class in diseased leaves with a relative abundance of 58.29% and were followed by *Proteobacteria* with 25.72% and only a 5.35% were unclassified bacteria. Furthermore, the most abundant genera were *Clavibacter*, *Pectobacterium*, *Propionibacterium* and *Staphylococcus*, that represented 45%, 15%, 9% and 8%, respectively.

Cultivable endophytic bacteria and their distribution in the tissues of the plant

We isolated and cultured endophytes from healthy plants and they were identified as *Gammaproteobacteria* (71%), *Actinobacteria* (12.5%), *Firmicutes* (12.5%) and *Alphaproteobacteria* (4%) (Fig. 5). In leaves, shoots and roots of healthy plants, the most abundant microorganisms were *Gammaproteobacteria*, and while *Actinobacteria* were isolated from shoots and roots, *Firmicutes* only were isolated from fruits samples, where they represented 60% of the community (Table 3).

Plants tissues with symptoms of gray spot on the tomato leaf

In diseased plants with gray spot leaf symptoms, the number of microorganisms isolated was smaller and were mostly

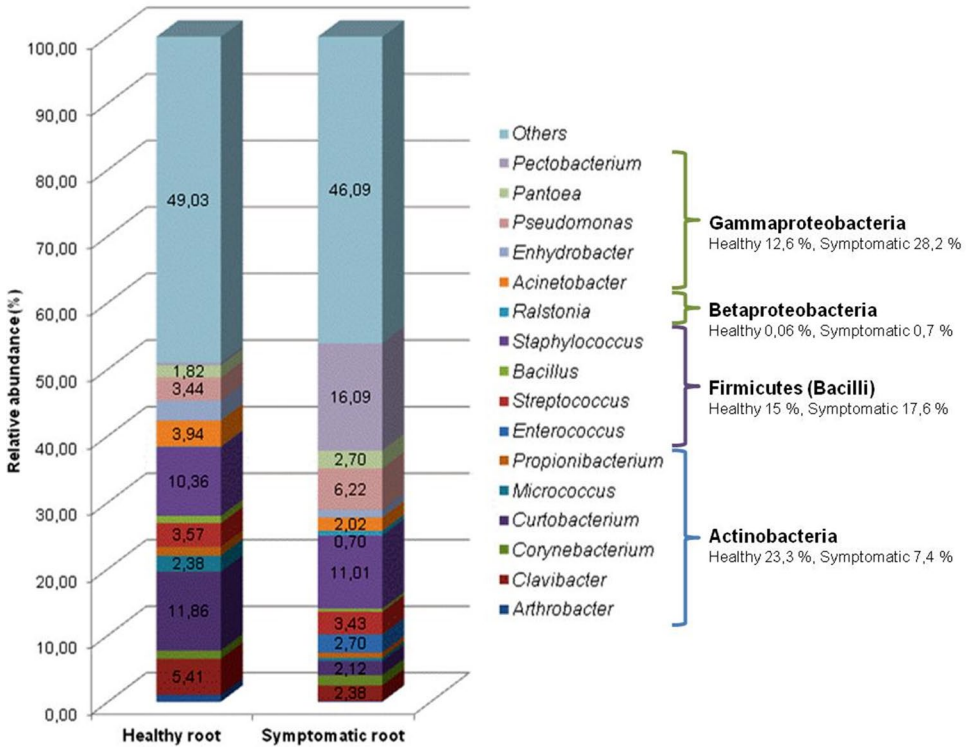

Fig. 3 Relative abundance of the main bacterial genera (> 0.5%) identified within healthy and symptomatic roots of Tomato. The genera with relative abundance < 0.5% and unclassified bacteria were grouped in "Others"

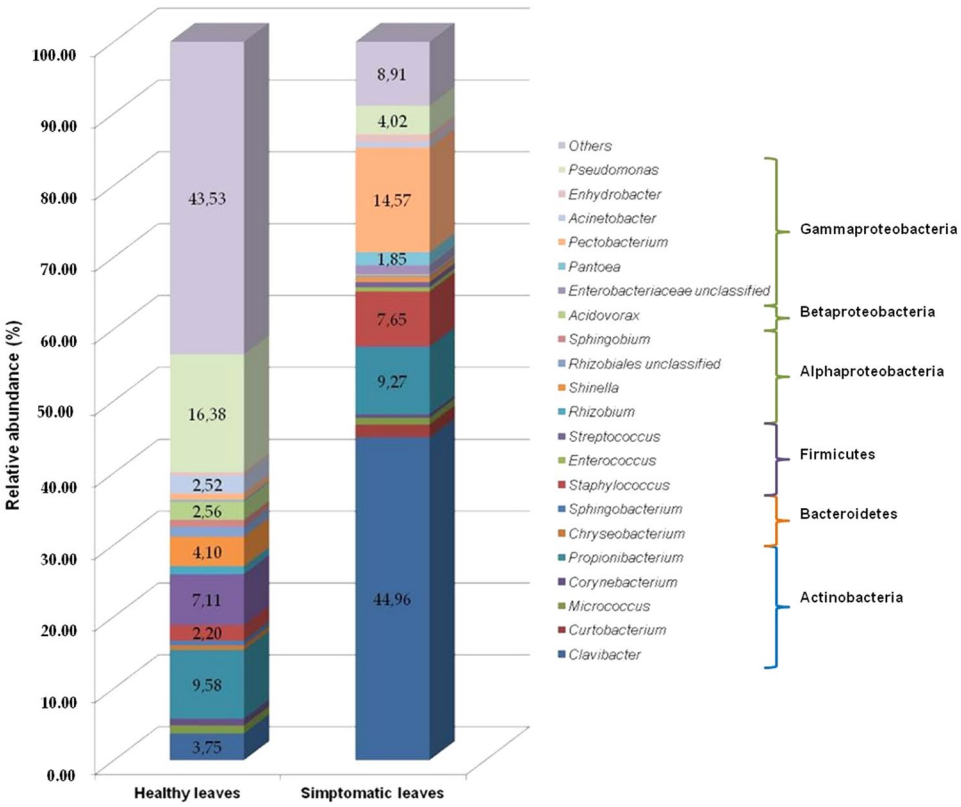

Fig. 4 Main bacterial (> 0.5%) genera and their relative abundance within healthy and symptomatic leaves of Tomato. The genera with relative abundance < 0.5% and unclassified bacteria were grouped in "Others"

Gammaproteobacteria in root, shoot and fruit samples. However, the biggest difference between bacterial endophytes of healthy and diseased tomato plants was found within leaves, since diseased leaves, unlike healthy ones, contained *Actinobacteria* as the most abundant microorganisms (Fig. 5). The genera of bacteria isolated from each plant organ are listed in Table 4.

Discussion

Endophytic bacteria, make-up part of plants microbiome, these microorganisms are associated to every single plant organ, where they are protected from environmental changes and therefore mostly might be affected by changes occurring within plant tissues (Ryan et al. 2008). Modifications within the endophytic communities might be due to the impact of the environment on the plant and as a result of this; endophytic bacteria might turn into a pathogenic phase, most probably due to their proliferation within plant tissue, which might change the internal homeostatic environment. Thus, changes provoked in the endophytic community of plants additionally might be affected by pathogens in an indirect manner as well as by other factors; in any case, it might be an index of plant health status perturbation and may result in a reduction in growth and yield.

The bacteriome of roots is less diverse than that of the rhizosphere and soil (Liu et al. 2017) and this is because roots prevent bacterial entry to the plant. Only a few lineages of soil bacteria colonize the root apoplast (Bulgarelli et al. 2012), like representatives of *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Ottesen et al. 2013; Liu et al. 2017). Interestingly, root samples of diseased and healthy plants presented a different endophytic community. *Actinobacteria* are inhabitants and stable components of the soil and seeds microbiomes, where they are relatively abundant (20 to 50%) depending on the cultivar genotype (López et al. 2018). Because of this, the vertical transfer is most probably the way bacteria move from the seed into the roots, which might be complemented by the entry of at least some of the microorganisms living in the soil (Li et al. 2014; Hardoim et al. 2015; Frank et al. 2017). In general, *Actinobacteria* are hardly affected by nutrient changes, which might occur along with germination, as a result of this *Actinobacteria* probably prevailed in roots. Roots of healthy plants contained more *Actinobacteria* than those of diseased plants, mainly bacteria of the genus *Curtobacterium* and *Clavibacter*. *Curtobacterium* is a genus that includes many plant pathogenic bacteria, as well as several species that either promotes plant growth or biocontrol pathogens by triggering plant defense responses (Lacava et al. 2007; Bulgari et al. 2011; Chase et al. 2016; Araújo et al. 2018). Healthy roots also contained *Clavibacter* suggesting that

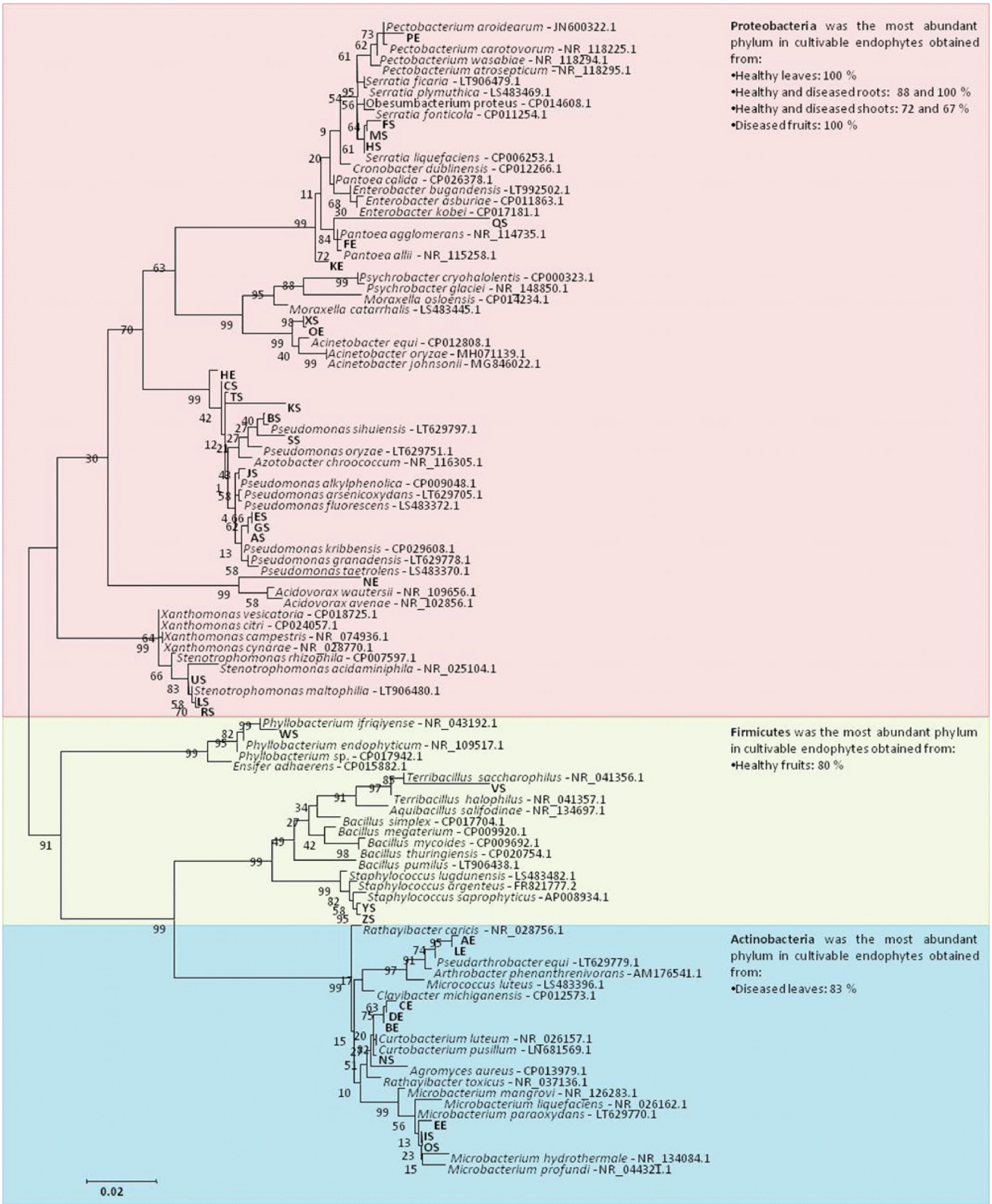

Fig. 5 Maximum likelihood tree inferred from the *16S rRNA* data set, of endophytic bacteria isolated from tissues of healthy and diseased tomato plants. The sequences generated in this study are in bold type letter. The numbers at the nodes represent bootstrap support values as a percentage of 1000 replicates. The scale bar represents the average number of nucleotide substitutions per site

Table 3 Identification of cultivable endophytic bacteria isolated from different tissues of healthy tomato plant using sequences of the *16S rRNA* gene

Isolate	Genus	Accession number	Sample
AS	*Pseudomonas* sp.	MH915620.1	Healthy leaves
BS	*Pseudomonas* sp.	MH915623.1	
CS	*Pseudomonas* sp.	MH915625.1	
ES	*Pseudomonas* sp.	MH915628.1	
FS	*Serratia* sp.	MH915630.1	Healthy roots
GS	*Pseudomonas* sp.	MH915631.1	
HS	*Serratia* sp.	MH915633.1	
IS	*Microbacterium* sp.	MH915634.1	
JS	*Pseudomonas* sp.	MH915635.1	
KS	*Pseudomonas* sp.	MH915637.1	
LS	*Stenotrophomonas* sp.	MH915639.1	
MS	*Serratia* sp.	MH915640.1	
NS	*Curtobacterium* sp.	MH915642.1	Healthy shoots
OS	*Microbacterium* sp.	MH915644.1	
QS	*Pantoea* sp.	MH915646.1	
RS	*Stenotrophomonas* sp.	MH915647.1	
SS	*Pseudomonas* sp.	MH915648.1	
TS	*Pseudomonas* sp.	MH915649.1	
US	*Stenotrophomonas* sp.	MH915650.1	
VS	*Terribacillus* sp.	MH915651.1	Healthy fruits
WS	*Phyllobacterium* sp.	MH915652.1	
YS	*Acinetobacter* sp.	MH915653.1	
XS	*Staphylococcus* sp.	MH915654.1	
ZS	*Staphylococcus* sp.	MH915655.1	

Table 4 Identification of cultivable endophytic bacteria isolated from different tissues of symptomatic tomato plant using sequences of the *16S rRNA* gene

Isolate	Genus	Accession number	Sample
AE	*Arthrobacter* sp.	MH915621.1	Symptomatic leaves
BE	*Curtobacterium* sp.	MH915622.1	
CE	*Curtobacterium* sp.	MH915624.1	
DE	*Curtobacterium* sp.	MH915626.1	
EE	*Microbacterium* sp.	MH915627.1	
FE	*Pantoea* sp.	MH915629.1	
HE	*Pseudomonas* sp.	MH915632.1	Symptomatic roots
KE	*Pantoea* sp.	MH915636.1	Symptomatic shoots
LE	*Arthrobacter* sp.	MH915638.1	
NE	*Acidovorax* sp.	MH915641.1	
OE	*Acinetobacter* sp.	MH915643.1	Symptomatic fruits
PE	*Pectobacterium* sp.	MH915645.1	

representatives of this saprophytic species might play a key role in plants (Zaluga et al. 2013; Yasuhara-Bell and Alvarez 2015), though some of them, like *Clavibacter michiganensis* subsp. *michiganensis* (*Cm* sbs. *m*) provokes a seed-borne disease of tomato, invading the xylem vessels, leading this to wilt, provoking lesions both on leaves as well as fruit and ultimately yield loss (Girish and Umesha 2005; De León et al. 2009). Surprisingly, some *Clavibacter michiganensis* sbs *michiganensis* isolated from tomato seeds as well as rice were not pathogenic (Cottyn et al. 2009). This raises a question regarding the role *Clavibacter* species play, which is particularly important considering that they belong to the tomato seed microbiome (López et al. 2018). Colonization of tomato plants by pathogenic *C michiganensis* sbs *m* is due to several virulence factors (Zaluga et al. 2013; Nandi et al. 2018) that are expressed in healthy tissues under certain conditions leading this to the development of disease symptoms. Unfavorable conditions might prevent disease development in tomato plants that might carry bacteria to other crops (Zaluga et al. 2013). In fact, saprophytic clavibacteria are widespread inhabitants of plants worldwide (Zinniel et al. 2002). Still, little is known concerning the role epiphytic and endophytic clavibacteria play in plants growth and development; particularly, regarding the effect, such populations have on disease development as well as growth. Our results suggest that either asymptomatic plants are the source of pathogenic bacteria that might behave as opportunistic pathogens or *Clavibacter* play other unknown roles in plant biology. However, the mechanisms and interactions, as well as the environmental signals that trigger such responses, remain unknown.

The relative abundance of many bacterial species suggests that they also might be involved in disease suppression by establishing a competition with pathogenic bacteria. The culture-independent approach used by Berendsen (Berendsen et al. 2012) found that *Gammaproteobacteria*, *Betaproteobacteria* as well as *Firmicutes* were involved in disease suppression (Berendsen et al. 2012). We found a higher relative abundance of *Pseudomonas* and a lower one of *Clavibacter* in tissues of healthy plants. Like with *Clavibacter*, the genus *Pseudomonas* also contains pathogenic as well as beneficial species. However, several *Pseudomonas* strains have been described that synthesize antimicrobial metabolites such as 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN), two compounds that were claimed to be responsible for the biological control exerted by *Pseudomonas* in different agroecosystems (Paulin et al. 2017; Nandi et al. 2018). DAPG is a broad-spectrum antimicrobial compound that also has been associated with plants induced systemic resistance, stimulation of root exudation and enhancement of root branching (Compant et al. 2005). In line with this, bacterial canker in tomato was reduced by *Pseudomonas* isolates that synthesize both DAPG and

HCN (Lanteigne et al. 2012). Furthermore, *Cm* sbs. *m* infection of tomato led to an increase in *Pseudomonas* that over-expressed the DAPG biosynthetic operon (Paulin et al. 2017). All this together, suggests that plants can secrete by themselves or through their interaction with organisms, molecules that might prevent pathogens development (Berendsen et al. 2012). However, the differences found in the composition of the bacterial community of roots appeared to be unrelated to the disease caused by *S. lycopersici,* which might be related with the plant organ that is affected by the pathogen that in this case mainly are leaves.

While analyzing the endophytic community within aerial tissues of gray leaf spot symptomatic plants, fruits presented the highest relative abundance and corresponded to the *Gammaproteobacteria,* however, the endophytic community was the same whether the fruit was from healthy or diseased plants, which may be because *Stemphylium* does not directly affect plant fruits. It has been reported that microorganisms associated with plants tissue produce secondary metabolites that either directly or indirectly affect locally fruit physiology (Droby and Wisniewski 2018). Regarding this, it would be interesting to study the effect of the endosphere microbiome of fruits on their susceptibility to pathogens particularly when they are stored.

Leaves have an enormous and diverse array of bacteria within their intercellular spaces. Romero et al. (2014) found that healthy tomato leaves contained a complex endosphere bacteriome composed mainly of five phyla that represented 99% of the population. *Proteobacteria* represented the most abundant phylum followed by *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia* and *Acidobacteria*. Ottesen et al. (2013) also found that *Proteobacteria* was the main component of tomato leaves microbiome. Our results confirmed that *Proteobacteria* was the predominant colonizer of healthy leaves, which probably was due not only to the relatively fast growth rate of the species within this phylum (Li et al. 2014) but also to the abundance of *Pseudomonas* sp., that might have prevented the development of *S. lycopersici* and therefore plants had no symptoms. Symptomatic leaves, on the contrary, had a lower abundance of *Proteobacteria*, which has already been observed in tomato roots infected by some soil-borne pathogens (Mendes et al. 2011; Li et al. 2014). The inhibition of *Proteobacteria* and particularly of *Pseudomonas* sp. in diseased leaves might be due to the profound impacts fungal pathogens had on the amount and diversity of nutrients as well as plant secondary metabolites within tissues, which in turn might have suppressed microorganisms particularly sensitive to environmental changes or secondary metabolites alterations (Cook et al. 1995). Interestingly, in diseased tissues, we found an increase in the relative abundance of bacteria belonging to the *Enterobacteriaceae* family, mainly *Pectobacterium* sp. that frequently coexists with pathogens (Erlacher et al. 2014). This *Enterobacteriaceae* are known for their capacity to degrade plant tissues. Both groups, accompanying organisms as well as those antagonistic of pathogens, should be considered in the development of plant protection strategies (Berg et al. 2017) while developing biotechnological tools to protect plants.

The largest modifications in the microbiome occurred in leaves of diseased tomatoes. Healthy leaves contained the greatest diversity of endophytic bacteria, which might be due to the wide array of nutrients and metabolites available that might favor growth and development of many diverse endophytic bacteria (Reiter et al. 2002). We hypothesized that the interaction of a more diverse population with the plant defense mechanisms might prevent diseases. Pathogen invasion might provoke changes within leaves microbiome and endophytes mostly involved in controlling pathogens (Erlacher et al. 2014), either by inhibiting expression of pathogenicity genes or by affecting the plant defense responses by triggering the synthesis of antagonistic molecules within plant tissue or plant´s surfaces (Fravel 1988). In line with this, we observed in symptomatic leaves changes in the endophytic bacterial diversity mainly of genera associated with antagonistic activity indicating that the disease might be the result of alterations of microorganisms' diversity. Previously, it has been suggested that stability of the microbial community and therefore recalcitrant action against invasion of intercellular spaces by pathogens is linked to its level of diversity (Jousset et al. 2011; Van Elsas et al. 2012) and the loss of certain groups of microorganisms might lead to the development of disease (Blaser 2014). The endophytic communities associated with symptomatic plant leaves were markedly lower in diversity and relative abundance compared to those in non-symptomatic plants. This could be due to the competition for nutrient sources and favorable niches for pathogens contributing in this way to a reduction in species richness and a reduction of evenness, leading this to a buildup of a certain population and finally to an unbalanced community (dysbiosis), at least compared to healthy plants (Bulgari et al. 2011). So most probably, the microbiome of each plant works as a shield, whose strength relays on diversity, which is crucial for the success of pathogenesis development and the establishment of biocontrol agents.

This preliminary analysis indicated that the bacterial communities associated with non-symptomatic plant leaves are more diverse and richer than symptomatic leaves. However, it remains to be determined if the decrease in richness was a direct effect of the pathogen or if the decrease in species evenness could be due to a defense mechanism against phytopathogenic agents, where the increase in the relative abundance of antagonistic species of pathogens is favored. Future studies designed at identifying the metabolic functions provided by the endophytic communities and the mechanisms that are triggered in host plants under different

circumstances are needed to elucidate the contribution of microbial population dynamics. In many cases, diseases are associated with dysbiosis or shifts which makes exploitation of the entire microbiome a desirable objective. Analyzing the plant microbiome as well as the metabolic interplay with the host plant opens new doors for advanced biocontrol technologies (Berg et al. 2017). In line with this, the genotype of the plant might exert a profound influence on diversity and richness of specific populations of microorganisms within the plant´s microbiome. The study of microbial endophytic communities entails considering, the highly variable and reactive environment provided by the living host (Robinson et al. 2010). For plants, modifiable environmental factors include soil type, temperature, humidity, and intensity and quality of light, in addition to biotic factors such as insects (Vorholt et al. 2017). While endophytes benefit from a substantially protected niche, they are nevertheless subject to changes in the host's physiology, which in turn responds to environmental stimuli (Zimmerman and Vitousek 2012). Campisano et al. (2017) found that differences were also observed between bacterial communities in the field and greenhouse-grown plants. This separation probably originates from the stability of greenhouse conditions and the inherent lack of fluctuations in temperature, wind, and humidity. Previous studies on the impact of environmental variables on soil microbial communities under controlled conditions (Kuffner et al. 2012; Zhang et al. 2013) have shown limited responsiveness of bacterial assemblies to minor temperature changes. It is known that temperature positively influence root exudation, and root exudates play an active role in recruiting microorganisms from the soil to the rhizosphere and, subsequently, to the endosphere (Broeckling et al. 2008). It is, therefore, conceivable that the increase in diversity and the alterations in the root-associated bacterial community depend on increased temperature (Campisano et al. 2017). Other relevant factors affecting endophytic communities are tomato cultivars and the environmental conditions like the climate. Dong et al. (2019) found that among the endophytic samples, both bacterial diversity and richness varied in different tissues, with the highest values in roots. However, in our study with healthy plants of tomato cultivar "Elpida" leaf tissues presented a larger diversity and greater richness, which was drastically modified by pathogens in diseased plants. While within the associated tomato bacterial community in cultivar "Zhongza 302″ and cultivar Elpida the most abundant phyla were Proteobacteria, at the genus level there were substantial differences. While some organisms might be occasional opportunistic ones as a result of stochastic events, there is growing evidence that the core community of microorganisms is consistently stable on healthy plants across space, time, and, in part, across organs, not only at the phylum level but also at higher taxonomic levels (Vorholt et al. 2017). Still, the relative abundances of individual phyla, classes, or genera might vary at least in part due to variations in the environment, plant genotypes, sampling locations, the temporal factors, and host plant blooming phenology (Bulgarelli et al. 2012; Lundberg et al. 2012; Ding and Melcher, 2016).The profile of the bacterial community allowed the description of the phylogenetic structure of the endophytic bacteria of tomato cv Elpida plant, while the functional perceptions derive largely from experiments with individual microorganisms. The culturable bacteria identified also were found within the microbiomes showed by high-throughput sequencing analysis. As expected, the isolates cultured in vitro belonged to the phylum with the greatest relative abundance in each organ of the plant. Although endophytes establish different types of relationships with their hosts, this is much more complex than thought before. Host-endophyte interactions are predominantly mutualistic ranging from mutualism to commensalism that includes latent and/or mild antagonism (Harrison and Griffin 2020). Furthermore, the influence of the taxa depends on the context (Rodriguez et al. 2009) which is crucial for the impact organisms or natural products synthesized and released by them have for the benefit of plants, in terms of growth promotion or pathogen biocontrol (Busby et al. 2017). Both basic and applied research concerning endophytes have been hampered by their unknown biodiversity and distribution within host plant and such a characterization is logistically difficult both in terms of obtaining pure cultures as well as sequencing (Carini 2019). Many studies confirmed that endophytic assemblies vary between tissue types (Haruna et al. 2018; Massoni et al. 2019), although no general patterns in endophytic richness have been described for tissue types. Adding greater complexity, some bacteria are endophytes of fungi (Harrison and Griffin, 2020), for them the most relevant habitat conditions might be those of the fungal host and not the plant. Another difficulty arises with endophytes that occur in low relative abundance at the time of obtaining pure cultures, although they probably contribute most to the endophytic biodiversity (Lynch and Neufeld 2015). Even though this study is far from elucidating the endophytic diversity in the tissues of the tomato plant, it is a starting point to begin the study of bacterial taxa within tomato and their contribution to plant growth and health, since we analyzed culturable and related them with the metagenomic analysis of plant tissue. This provided a cleared panorama of the tomato endophytes, an approach to begin the study of bacterial taxa with significant contributions to plant health and growth and is closely related to more complete sight provided by the metagenomic analysis of the tissues.

Research with crop-dependent techniques is crucial for the study and future biotechnological use in biological control; still, a microbiological research approach should be focused on the complete biological system, to understand

the interactions in the context they occur. This would be a good starting point for the isolation of crucial groups of bacteria that might be responsible for a phenotype of interest (Bulgarelli et al. 2013, Finkel et al. 2017, Vorholt et al. 2017, Jansson and Hofmockel 2018). The main advantage of meta-omic approaches is the possibility of identifying microbial traits in a plant microbiome without cultivating its members. The limitations arise due to the complexity of the microbiota of the plant and its inherent traits (Bulgarelli et al. 2013). Ultimately, it is fair to underline that metabarcoding provides first observations or indirect correlations and additional experiments are needed to reveal whether diversity within plant microbiome is a cause or consequence of an observed plant phenotype. The microbiome associated with the plant is emerging as a fundamental feature of plant growth and health (Bulgarelli et al. 2013; Vorholt et al. 2017). Schlaeppi and Bulgarelli (2015) stated that the combined study of the plant microbiome and innate immune functions will provide lasting and sustainable protection against diseases since they will provide tools for controlling the burden of pathogens.

In conclusion, we showed that only a few taxa of bacteria inhabiting tomato plants could be cultured and that all plant organs have a highly diverse endophytic bacterial, whose activity might affect plant growth and development as well as health. The roots seem to be an important barrier for microbes and leaves appear to be the organs with the higher diversity which is incidentally related to plant health. Fruits also contain a complex bacterial community that appeared to be unaffected by foliar diseases such as gray leaf spot at least under the conditions studied.

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