

Exploratory profiling of microbial communities associated with tapping panel dryness in *Hevea brasiliensis*

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Abstract

Tapping Panel Dryness is a complex physiological disorder in *Hevea brasiliensis* that leads to the cessation of latex flow, causing significant economic loss, yet its underlying cause remains unclear. Anatomical investigation of bark samples collected from TPD-affected samples exhibited deformed latex vessels, blocked sieve tubes, and DNA-containing bodies within phloem elements. Metagenomic profiling indicated largely similar microbial composition and diversity between healthy and TPD-affected bark samples, except for the presence of low-abundance taxa such as phytoplasma only in affected samples. However, predicted metabolic pathways differed significantly between healthy and TPD samples. The combined anatomical, cytological, and molecular evidences in the current study supports the potential involvement of phytoplasma in the etiology of TPD.

1. Introduction

Hevea brasiliensis, also known as Para rubber tree, belonging to the Euphorbiaceae family, is indigenous to the Amazonian rainforest. Currently, it is cultivated worldwide for its latex, which is a major source of natural rubber, an important raw material for the commercial industry. More than 35,000 products are synthesized from natural rubber, including gloves, automobile and aircraft tires.

Synthetic rubber cannot match natural rubber's exceptional properties, including tensile strength, elasticity, thermal and electrical insulation, and resistance to abrasion and wear and tear, as well as resilience (Kawahara et al. 2022). The market for natural rubber continues to expand due to rising demand from automotive sectors and growing interest in sustainable rubber sources. This demand supports the livelihood and economy of millions of smallholder farmers who contribute to rubber production. Thus, the production and productivity of natural rubber plays a decisive role in the economic growth and development of rubber-growing countries (Thangamalai 2021; Puskas et al. 2024).

Globally, natural rubber is synthesized from the latex of *H. brasiliensis*. Latex is a milky white colloidal solution consisting of water (60%), cis-1,4-polyisoprene (35%), and non-isoprene molecules (5%) such as proteins, lipids, carbohydrates, and minerals (Bottier 2020). Specialized laticifer cells in the bark, leaves, and petioles of the rubber tree synthesize this valuable substance. Commercial latex extraction occurs through a process called tapping, which involves making a controlled incision through half the trunk's circumference at a 30° angle. This precise cut exposes latex vessels in the inner bark while avoiding damage to the cambium. Latex exudes from these incised laticifers and flows along the tapping panel, the channel created by this incision, into collection cups (Wang et al. 2023). While this method is designed to ensure sustainable production without harming the tree, repeated tapping can sometimes lead to physiological stress and health issues in the tree. One of the most significant challenges faced in this context is Tapping Panel Dryness (TPD), a condition where specific sections or entire tapping panel cease to produce latex despite regular tapping (Herlinawati et al. 2022).

Unlike occasional dryness caused by improper tapping or temporary stress, TPD syndrome is more persistent due to irreversible cessation of latex flow and bark necrosis (Herlinawati et al. 2022). This

condition substantially reduces a tree's productive lifespan through permanent blockage of latex biosynthesis and tissue death, severely impacting natural rubber production worldwide.

TPD manifests as a complex syndrome rather than a disease with a single identifiable cause. Despite extensive research, its precise etiology remains elusive. While some studies suggest that the severity of TPD is dependent on the rubber tree clone (Tistama et al. 2019), the heritability of this trait has not been conclusively established by geneticists and is contested by conflicting reports of environmental stress and overexploitation (Herlinawati et al. 2022). The clustering pattern of TPD-affected trees observed in plantations and gradual spread of symptoms to adjacent trees over time (Abraham et al. 2013), raises the possibility of infectious agents associated with the development of the syndrome. Investigations into potential pathogenic agents, including viruses and viroids, have not provided definitive evidence of their involvement in TPD (Ramachandran et al. 2000; Zhao et al. 2023). Studies have reported bark abnormalities such as scaling, cracking, and necrotic streaking in association with TPD, yet comprehensive investigations have failed to implicate any fungal, bacterial, viral, or protozoan agents responsible for its onset (Pellegrin et al. 2004). This persistent knowledge gap necessitates alternative investigative approaches.

Recent systematic investigation from the symptomatology, anatomical studies using confocal and scanning electron microscopy, followed by investigations on physiological changes in the TPD affected bark tissues, strongly suggested the possible presence of phytoplasmas (Philip et al. 2025). Hence, considering the fundamental role of prokaryotic communities in plant health and disease progression, this study was carried out to verify the phytoplasma presence and to determine whether composition of prokaryotic community in bark tissue differs between healthy and TPD-affected samples.

2. Materials and methods

2.1. Collection of samples and study site

Bark samples were collected from a Tapping Panel Dryness (TPD)-affected plantation of clone RR11 430 (Fig. 1a) maintained by the Rubber Research Institute of India (RR11), Kottayam, Kerala, India (9.568689 N, 76.574478 E). The plantation had a TPD incidence rate of approximately 20%, and the trees were 15 years old, with no specific manurial treatments applied. Tapping was conducted using the S2d3 system, where the bark was cut spirally through half the circumference (S2) of the trunk once every three days (d3).

Experimental trees exhibiting typical symptoms of TPD (Fig. 1b & 1c), such as complete blockage of latex flow, scaling, and peeling of bark, were selected as diseased samples. Healthy samples as control were collected from trees with intact bark, regular latex flow, and no visible symptoms of dryness (Fig. 1d). Bark samples collected from healthy and TPD affected trees were subjected to anatomical and molecular investigation.

2.2 Anatomical investigation of healthy and TPD-affected bark samples

Tangential longitudinal sections with an approximate thickness of 35 μm were prepared using a sliding microtome from the inner soft bark region of *H. brasiliensis*, a tissue rich in sieve tubes and latex vessels. The sections were then used to examine the presence of callose deposition, latex localization, putative phytoplasma detection, and ultrastructural alterations associated with tapping panel dryness (TPD) syndrome using a combination of histochemical stains, light microscopy, confocal laser scanning microscopy (CLSM), and scanning electron microscopy (SEM).

2.2.1 Latex vessel staining using Oil Red O

Oil red O based staining was used to identify latex-bearing cells in *Hevea* (Hamzah et al. 1988). Tangential longitudinal sections were treated with 60% isopropanol for 5 minutes followed by staining with 0.5% Oil Red O (w/v) (prepared in 100% isopropanol and diluted to 60% before use). Sections were incubated in the stain for 30 minutes, rinsed briefly in 60% isopropanol, and then mounted in 50% glycerol. Observations were made under a bright field light microscope (LEICA DM 2500 LED).

2.2.2 Callose staining with Congo Red

To assess callose deposition in sieve tube elements, sections were stained with 0.1% (w/v) Congo Red (Hi Media, India) for 15 minutes in the dark at room temperature (Sivan et al. 2011). After two washes with distilled water, stained sections were mounted in distilled water and examined under a bright field light microscope (LEICA DM 2500 LED)

2.2.3 CLSM based detection of putative phytoplasma in the sieve tubes of bark sample

Sections were fixed in 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS; pH 7.2) for 30 minutes at room temperature, washed thoroughly with PBS (3 $\times$ 5 min), and stained with 1 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole (DAPI) (Hi Media, India) for 20 minutes in the dark (Andrade and Arismendi 2013). After washing in PBS, samples were mounted in 50% glycerol and examined using a confocal laser scanning microscope (CLSM; Leica TCS-SP2, Rajiv Gandhi Centre for Biotechnology, Trivandrum, India) equipped with a UV laser (excitation at 358 nm, emission collected at 461 nm). Images were acquired using Leica Application Suite Advanced Fluorescence software (version 2.0).

2.2.4 Scanning electron microscopy (SEM) based ultrastructural detection of sieve tubes

Tangential longitudinal bark sections were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours at 4°C, post-fixed in 1% osmium tetroxide for 1 hour, and dehydrated in an ascending ethanol series (30% to 100%) (Philip et al. 2025). Samples were critical-point dried using a BAL-TEC CPD

030 Critical Point Dryer (Rajiv Gandhi Centre for Biotechnology, Trivandrum, India) and mounted on aluminium stubs using double-sided carbon tape. Specimens were sputter-coated with gold in a BAL-TEC SCD 005 sputter coater and examined using a JEOL JSM-6390LV scanning electron microscope operated at 10 and 15 kV.

2.3 Metagenomic analysis of prokaryotic communities in healthy and TPD-affected bark samples

Total genomic DNA were isolated from bark tissues of healthy and TPD-affected trees and used for 16S rRNA gene-based library preparation and high-throughput sequencing to profile the associated microbial communities. For each condition (healthy and TPD-affected), two trees were selected as biological replicates.

2.3.1 DNA extraction from the bark samples of healthy and TPD affected trees

Bark samples from multiple portions of the trunk were processed by first removing the outer cortex and grinding the remaining tissue in liquid nitrogen to obtain a fine powder. DNA extraction was performed using a slightly modified version of the protocol described by (Doyle and Doyle 1987). Approximately 2 g of ground tissue was mixed with preheated CTAB buffer (2% CTAB, 1% polyvinyl pyrrolidone, 1.4 M sodium chloride, 50 mM EDTA, 100 mM Tris-HCl at pH 7.8, and 0.2% β -mercaptoethanol). The lysate was incubated at 65°C for 30 minutes, followed by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifugation at 15,000g for 25 seconds. The supernatant was carefully collected, treated with 4 μ l of RNase, and incubated at 37°C for 30 minutes. DNA was precipitated by adding two-third volume of ice-cold isopropanol. The mixture was centrifuged at 15,000g for 25 seconds to recover the pellet. The resulting pellet was washed with 70% ethanol, air-dried, and dissolved in Milli-Q water. The quality and quantity of the extracted DNA were assessed using agarose gel electrophoresis and Invitrogen Qubit 4 Fluorometer for suitability in PCR-based amplification of the 16S rRNA gene. Metagenome sequencing, library preparation and bioinformatic analysis were done with the help of commercial sequencing service providers Eurofins Genomics India, Bengaluru and CloneGtyping, Bengaluru, both from India.

2.3.2 Preparation of 2 x 300 MiSeq library

The amplicon library was prepared using the Nextera XT Index Kit. Primers targeting the bacterial-specific region were designed by Eurofins Genomics Lab, India (forward primer: 5' GCCTACGGGNGGCWGCAG 3' and reverse primer: 5' ACTACHVGGGTATCTAATCC 3'). Quality-checked (QC) amplicons with Illumina adapters were further amplified using i5 and i7 primers (Illumina 2013), which incorporate multiplexing index sequences and common adapters (P5 and P7) (Illumina 2013) essential for cluster generation. The amplicon library was purified using AMPure XP beads and quantified with a Qubit Fluorometer. Amplified libraries underwent fragment analysis on the 4200 TapeStation system (Agilent Technologies) using D1000 ScreenTape, following the manufacturer's

protocol. Libraries passing QC were sequenced on the MiSeq platform. The resulting sequences from this study have been deposited in the Sequence Read Archive (SRA) under BioProject ID: PRJNA1203434.

2.3.3 Sequence processing, statistical analysis and functional prediction

The raw data quality was assessed using the FastQC tool (S. Andrews 2010). The reads were imported into the QIIME2 environment (Bolyen et al. 2019) *via* the qiime tools import function. After importing, the reads were merged to form a consensus sequence, and a Phred score of 20 was applied as the quality filtering parameter for downstream analyses. Subsequent quality control steps included denoising and dereplication using the qiime vsearch dereplicate-sequences function (Rognes et al. 2016), which collapsed identical sequences into unique representative sequences. Chimeric sequences were identified and removed using the qiime vsearch uchime-denovo function (Edgar et al. 2011). Operational Taxonomic Units (OTUs) were clustered de novo at 97% sequence similarity using the qiime vsearch cluster-features-de-novo command, grouping similar sequences into OTUs that represent distinct bacterial taxa. The OTUs were classified using the SILVA 138.1 database. The qiime feature-table summarize function was then used to generate the feature/OTU table.

For further analysis, the Microeco package (Liu et al. 2020) (<https://github.com/ChiLiubio/microeco>) was utilized. The relative abundance of the top 10 phyla and 12 genera was calculated and visualized using boxplots. Alpha diversity metrics, including observed species, Chao1, ACE, Shannon, Simpson, and Inverse Simpson indices, were computed, with significant variations determined by a t-test ($p < 0.05$).

To predict the functional potential of the microbial communities, PICRUSt2 (Douglas et al. 2020) was employed. Representative sequences in FASTA format and the OTU table (BIOME/TSV format) were exported from QIIME2 for input. The pipeline began by placing Amplicon Sequence Variants (ASVs) onto a reference phylogeny using Hidden-State Prediction (HSP). Gene family abundances, including KEGG Orthologs (KO) and EC numbers, were predicted based on the phylogenetic positioning of the ASVs, and pathway-level predictions were made using MinPath. PICRUSt2 further normalized these predictions to account for variations in 16S rRNA gene copy numbers. The predicted gene families and metabolic pathways were analyzed and visualized using STAMP (Parks et al. 2014), providing insights into the functional potential of the microbial communities.

3. Results

3.1 Anatomical differences in the healthy and TPD-affected bark samples of *H. brasiliensis*

To understand the anatomical changes associated with Tapping Panel Dryness (TPD) in *H. brasiliensis*, comparative observations were made between healthy and TPD-affected bark tissues, particularly

focusing on sieve tubes, latex vessels, and the presence of intracellular phytoplasma-like bodies. The following subsections detail the findings from each method.

3.1.1 Visualization of latex vessel organization in healthy and TPD-affected bark samples

In healthy bark sections, laticiferous tissues appeared as well-defined, regularly aligned structures, with uniform red staining (Fig. II a). The Oil Red O selectively stained lipid-rich latex vesicles, which were densely packed within the laticifers, indicating normal rubber biosynthesis and storage activity. In contrast, bark tissues from TPD-affected trees (Fig. II b) exhibited noticeable reduction in Oil Red O staining intensity with deformed latex vessel distribution. Dark greyish discoloration was observed in ray parenchyma cells and latex vessels indicating impaired rubber biosynthetic activity or altered metabolic processes associated with the onset of TPD.

3.1.2 Callose deposition in sieve tubes of healthy and TPD-affected bark samples

Sections stained with Congo red were observed for callose deposition. Marked differences were observed between healthy and TPD-affected samples stained with Congo red under bright-field light microscopy. At lower magnification (10X), prominent red staining was localized in the sieve plates of TPD-affected sample (Fig. III). At higher magnification (40X), discrete red bands were observed occluding sieve pores, indicating dense callose deposits across multiple sieve elements (Fig. IV). These occlusions were largely absent or minimal in healthy samples, where sieve plates appeared clear and structurally intact (Fig. IV), indicating free axial and radial transport in the sieve tube.

3.1.3 DAPI fluorescence microscopy reveals nuclear material in sieve tubes of TPD-affected sample

Bark sections stained with DAPI, were examined to detect the presence of nuclear material in sieve elements. In healthy bark tissues, no fluorescence signal was observed, indicating the absence of extranuclear DNA in sieve tube elements (Fig. V). In contrast, sections from TPD-affected samples showed prominent DAPI fluorescence localized within sieve elements. These bright, punctate signals suggest the presence of DNA-containing bodies, or microbes potentially corresponding to phytoplasma.

3.1.4 Scanning Electron Microscopy (SEM) of sieve tube of TPD-affected bark sample

SEM imaging of TPD-affected bark sections, at 10 and 15 kV revealed the presence of pleomorphic bodies distributed in the sieve tube at a size ranging from 372 to 991 nm (Fig. VI).

3.2 Composition, diversity and functional potential of prokaryotic communities in healthy and TPD-affected

samples

Metagenomic analysis of bark tissues from healthy and TPD-affected *H. brasiliensis* trees provided insights into the prokaryotic community structure, diversity, and predicted functional potential, with results described in the following subsections.

3.2.1 Quantitative and qualitative analysis of DNA and amplicon libraries from healthy and TPD-affected bark samples

The DNA yield varied across samples, with healthy and TPD affected bark yielded an average of 128.1 and 59.8 ng/μL, respectively. The purity of all DNA samples was within an acceptable range (260/280 ratio ~ 1.8), ensuring their suitability for amplicon library preparation. Amplification of 16S rRNA generated high quality amplicons of an average size 595 bp which was standardized to 19 ng/μL for sequencing.

3.2.2 Sequencing overview and taxonomic profiling of healthy and TPD-affected bark microbiomes

Sequencing of amplicon libraries generated a substantial number of raw reads, which were subsequently quality filtered to obtain high-confidence sequences of ~ 301 bp, for taxonomic analysis. In healthy samples, a total of 1,52,547 quality (HQ) reads yielded 2,111 operational taxonomic units (OTUs). The classification of these sequences identified 30 phyla, 93 class, 174 order, 310 family and 500 genera. In contrast, TPD-affected samples exhibited a total of 1,69,807 reads with 2,224 OTUs identified. Their classification revealed a larger microbial diversity in TPD-affected samples, comprising 30 phyla, 92 class, 175 order, 313 family and 505 genera.

3.2.3 Comparative analysis of bacterial communities in healthy and TPD affected samples

Microbial community composition showed no significant differences between healthy and TPD-affected bark samples at any taxonomic level ($p > 0.05$). Bacteria and Archaea were present in similar proportions, with Proteobacteria and Actinobacteriota as dominant phyla in both conditions. Differences in relative abundances across the major taxa were not statistically significant (Fig. VII, S1&S2). However, a few prokaryotes namely, *Comamonas*, *Niastella*, *Phytoplasma*, *Salinibacter*, and *Wolbachia* were detected exclusively in TPD-affected samples, albeit at very low abundances ($< 0.01\%$) (Table. I).

3.2.4 Diversity analysis and richness estimates

Alpha diversity of the samples was analyzed using various diversity indices like Simpson, Chao, Shannon-Wiener, Abundance-based Coverage Estimator (ACE) index, Inverse Simpson (Fig. VIII). Species richness was analyzed using Simpson diversity index and Shannon index. Richness of rare species was

estimated by Chao 1 and ACE index. Diseased samples exhibited higher variation across all indices as indicated by larger spread in box plots. Consistently lower indices in healthy samples suggest uniformity of microbial community. However, statistically these differences between healthy and infected samples resulted not significant ($p > 0.05$).

3.2.5 Predicted pathways from 16S rRNA

A total of 7418 PICRUSt2 predicted KEGG orthologs (enzymes) were collapsed to 426 KEGG pathways. The analysis revealed significant alterations in various metabolic pathways between healthy and infected individuals (Fig. IX). In a broader way of categorization in healthy samples, pathways involving amino acid biosynthesis, glycan biosynthesis and metabolism and carbohydrate metabolism resulted highly enriched. Among them, superpathway of methylglyoxal degradation (glycan biosynthesis) and Entner-Doudoroff pathway III (carbohydrate metabolism) were significantly abundant ($q < 10^{-15}$).

Among the top 25 differentially abundant pathways, the superpathway of bacteriochlorophyll a biosynthesis and isopropanol biosynthesis exhibited the most pronounced increase in the infected group, with a significant difference between each other ($q < 10^{-15}$). Additionally, pathways related to formaldehyde assimilation (serine pathway), chlorosalicylate degradation and protocatechuate degradation also showed significant overrepresentation in infected individuals. This suggests enhanced microbial biodegradation of xenobiotics or host metabolites.

4. Discussion

This study combined anatomical, cytological, metagenomic, and molecular approaches to investigate potential biotic associations with TPD, with a specific focus on vascular tissue integrity and microbial community dynamics. The multidisciplinary findings of the study propose a unified model of TPD pathogenesis and highlights the significance of phytoplasma association in TPD-affected trees.

Latex flow in *H. brasiliensis* is highly dependent on the structural and functional integrity of laticifers and their associated phloem tissues (De Fay 2011). Latex vessels stained with Oil red O in the healthy samples appeared structurally intact. As Oil red O has strong affinity for neutral lipids, uniform and intense red staining in healthy samples, indicate abundant and well-preserved lipids in the laticifers (Hamzah et al. 1988).

Congo red staining of tangential bark sections from healthy *H. brasiliensis* revealed well-organized sieve tube elements with clearly visible perforated sieve plates. The sieve plates, which form the interface between adjacent sieve elements, appeared open and unobstructed, allowing unimpeded transport of photosynthates and signaling molecules. Latex vessel absorbs nutrients from neighboring sieve tubes for latex biosynthesis (Sivan et al. 2011). The absence of callose or phenolic deposition in healthy sample suggests that the phloem is physiologically active and free of stress-induced blockages, maintaining uninterrupted nutrient supply to neighboring tissues, including latex vessels (De Fay 2011).

Hence, disruption in sieve tube function directly impacts the metabolic activity of adjacent laticifers (Sivan et al. 2011). Congo Red, a diazo dye with specific affinity for β -1,3-glucans (Piccinini et al. 2024), revealed prominent callose deposition and blockage in the sieve plates of TPD-affected bark. Such blockages impair both radial and axial transport in the phloem, leading to nutrient deprivation in latex vessels. This, in turn, disrupts rubber biosynthesis and promotes metabolic stress. Anatomical symptoms such as reduced lipid staining, deformation of latex vessels, and darkened cell contents in TPD-affected samples are consistent with previous studies linking nutrient starvation and oxidative damage in latex vessels due to sieve tube dysfunction (Sivan et al. 2011; Zhang et al. 2016). Stress in sieve tubes, whether from biotic or abiotic origins, can thus severely impact latex production. DAPI staining in confocal laser scanning microscopy (CLSM) revealed fluorescence exclusively in TPD-affected samples. As sieve tubes are anucleate and DAPI binds to AT-rich DNA, this fluorescence strongly suggests the presence of microbial DNA, most plausibly phytoplasma, known to inhabit sieve tubes and possess AT-rich genomes (Andrade and Arismendi 2013). SEM further confirmed the presence of pleomorphic bodies (319–990 nm) resembling phytoplasmas in TPD-affected samples. These findings align with previous report of association of phytoplasma with TPD-affected trees of *H. brasiliensis* clone RR11 105 (Philip et al. 2025).

16S rRNA based metagenomic analysis was done to understand the overall prokaryotic composition and diversity in healthy and TPD-affected sample. The bacterial community composition remains consistent across healthy and TPD-affected samples, with no significant shifts in dominant microbial groups at higher taxonomic levels. At the kingdom level, both healthy and TPD-affected samples exhibited similar proportions of Bacteria and Archaea. Various other plants like rice, maize and Scots pine also have reported to be associated with several groups of Archaea in their phytobiome (Jung et al. 2020). Proteobacteria and Actinobacteria were the most abundant bacterial phyla in both samples. These major phyla are commonly present in almost all ecosystems. Acidobacteria, Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes are dominant phyla reported from bark of trees like avocado and red oak (Aguirre-von-Wobeser 2020; Hudson et al. 2023).

The microbial community composition at all taxa remains largely similar in healthy and TPD-affected samples. This suggests that the overall microbiome composition may not be heavily impacted by the disease, or that the disease is not directly associated with large-scale microbial shifts detectable through 16S rRNA amplicon sequencing. Amplicon sequencing, which targets conserved regions (*e.g.*, 16S rRNA), may not be sensitive enough to detect functional differences or low-abundance microbes that could play a critical role in disease development (Rizal et al. 2020).

Of particular interest is the presence of phytoplasma, with a very low concentration exclusively detected in TPD affected samples. Although detected at extremely low concentrations, phytoplasmas possess the potential to alter host plant metabolism profoundly. Previous studies have shown that concentrations as low as 370 to 34,000 cells per g. of tissue is associated with symptoms of witches' broom in apple trees, with nested PCR detecting as few as 4 to 340 cells in reaction mixtures (Berges et al. 2000). Diseases like, elm yellows, peach X disease, apple proliferation, arecanut lethal yellowing and grapevine bois noir

are some of the examples of disease associated with phytoplasma presence in perennial crops with serious economic impact (Wei et al. 2022; Wang et al. 2024).

Furthermore, the minimal concentration of phytoplasmas might explain their low abundance in metagenomic sequencing data, while a possible role in pathogenesis emphasizes their ability to induce disease without significantly altering the microbial diversity or composition in the host microbiome. Recent studies indicate that not all infections lead to major changes in microbial composition. In some cases, like strawberry with signs of plant stress or pathogens, the microbiome showed limited variation between healthy and diseased plants, with only a few taxa displaying noticeable shifts. This suggests that even under pathogenic stress, a stable core microbiome may be retained (Hassani et al. 2023).

Phytoplasmas are prokaryotes lacking rigid cell wall. However, their genome size is greatly reduced as a result of reductive evolution from Gram-positive bacteria belonging to Firmicutes. Lack of several genes for basic metabolic pathways and dependence on sucrose as the main source of carbon made them an obligate plant pathogen inhabiting phloem sieve tubes (Oshima 2021). Given that sieve tubes are rich in sucrose, a precursor for rubber biosynthesis, their blockage due to thick callose and P-protein deposition of TPD-affected trees hint the potential role of phytoplasmas in inducing the syndrome (Sivan et al. 2011; Buxa-Kleeberg et al. 2015). Plant defense mechanisms, such as callose deposition and aggregation of P-protein filaments in sieve plates, are reported in plants infected by phytoplasmas (Gallinger and Gross 2020; Wei et al. 2022). Nutrient deprivation, coupled with stress-induced defense signaling, could trigger hypersensitive responses and programmed cell death in laticifers, ultimately disrupting latex biosynthesis (Zimmermann et al. 2015; Meisrimler et al. 2021). By linking phytoplasma detection to characteristic symptoms like callose deposition, sieve tube blockage, and laticifer shrinkage, highlights its potential as a critical pathogen underlying TPD, even at low concentrations.

Analysis revealed that there is no significant variation in microbial diversity indices for healthy and TPD-affected samples. This implies that TPD may not be heavily impacted by the microbial richness and evenness. Amplicon metagenomics often capture overall community composition wherein low titre and localized microbes' effect on richness, evenness and microbial community structure gets unaffected (Cena et al. 2021). Well supported by the information on symptomatology, pattern of spread, and anatomical changes which are obtained through other experiments in this study, the data on metagenomics suggests or rather detects biologically meaningful trends (Dahlberg et al. 2023) indicating the presence of phytoplasmas in TPD.

Despite the similar microbial diversity and composition, there is a significant difference in the metabolic pathways enriched in healthy and TPD affected samples. This could possibly be due to calculation of diversity metrics at a broad taxonomic level (*e.g.*, genus or species), and they may not be sensitive enough to detect strain-level or functional differences that are important for pathway prediction. However, the metabolic pathways identified in this study were predicted using PICRUSt, which infers functional potential based on more detailed taxonomic resolution or phylogenetic relationships (Sun et

al. 2020; Toole et al. 2021). While these results provide valuable insights into potential microbial metabolic shifts, they do not represent direct biochemical activity.

Pathways involved in phenolic compound degradation, such as methylgallate degradation, gallate degradation, and chlorosalicylate degradation, were significantly enriched in diseased samples. These compounds are known to play a role in plant defense, and their increased degradation may represent a counter-defense mechanism by pathogens to neutralize plant defenses and establish infections (Zhang et al. 2020, 2022; Soal et al. 2022). Phytoplasma infections are known to interfere with host secondary metabolism, particularly phenylpropanoid and salicylic acid pathways, which could indirectly influence microbial functional dynamics (Pradit et al. 2019; Bauters et al. 2021; Dermastia et al. 2023).

5. Conclusion

This study presents a multidisciplinary investigation into the possible biotic factors associated with TPD in *H. brasiliensis*. While there are no major shifts in the dominant microbial groups between healthy and TPD-affected bark, the functional insights from pathway analysis, particularly the enrichment of biodegradative pathways in TPD-affected samples, open avenues for further exploration into the role of microbial metabolism in disease dynamics and possible microbial interactions with host metabolites. Lack of amplification of rare taxa is one of the limitations of amplicon based metagenomic sequencing and hence make it difficult to draw a conclusion on the abundance of microbes. Anatomical and cytological alterations in phloem tissues, alongside metagenomic and PCR based evidences, suggest the presence of a divergent phytoplasma strain in the TPD-affected samples. Although at low concentration, the detection of phytoplasma sequences could be significant, suggesting their potential pathogenic role in TPD. Further studies using targeted and deeper sequencing techniques will elucidate its potential contribution to disease development. Current investigation provides preliminary insights into the biotic complexity of TPD and lay groundwork for future confirmatory studies.

Declarations

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used free version of Claude in order to improve the writing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Author Contribution

A.T. conducted experiment and wrote manuscript text. S.K.P. and S.P. structured the research work and reviewed the manuscript. All other authors reviewed manuscript

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Figures

Fig. I



Figure 1

(a) Rubber plantation (b) Dried panel (c) TPD-affected rubber tree (d) Healthy rubber tree

Fig. II

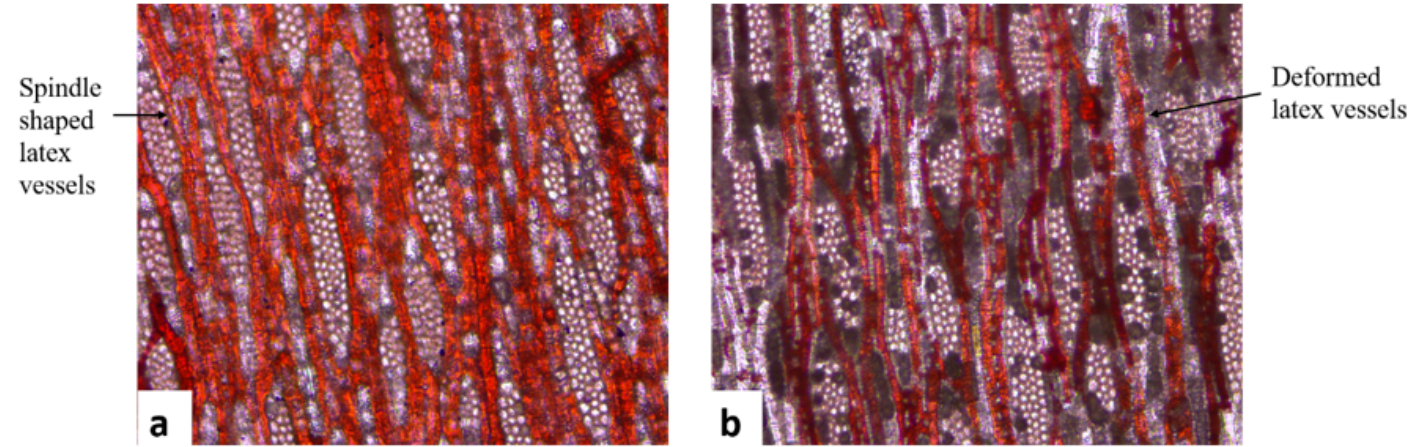


Figure 2

(a) Latex vessels in healthy sample (b) Latex vessels in TPD-affected sample; (10X magnification)

Fig. III

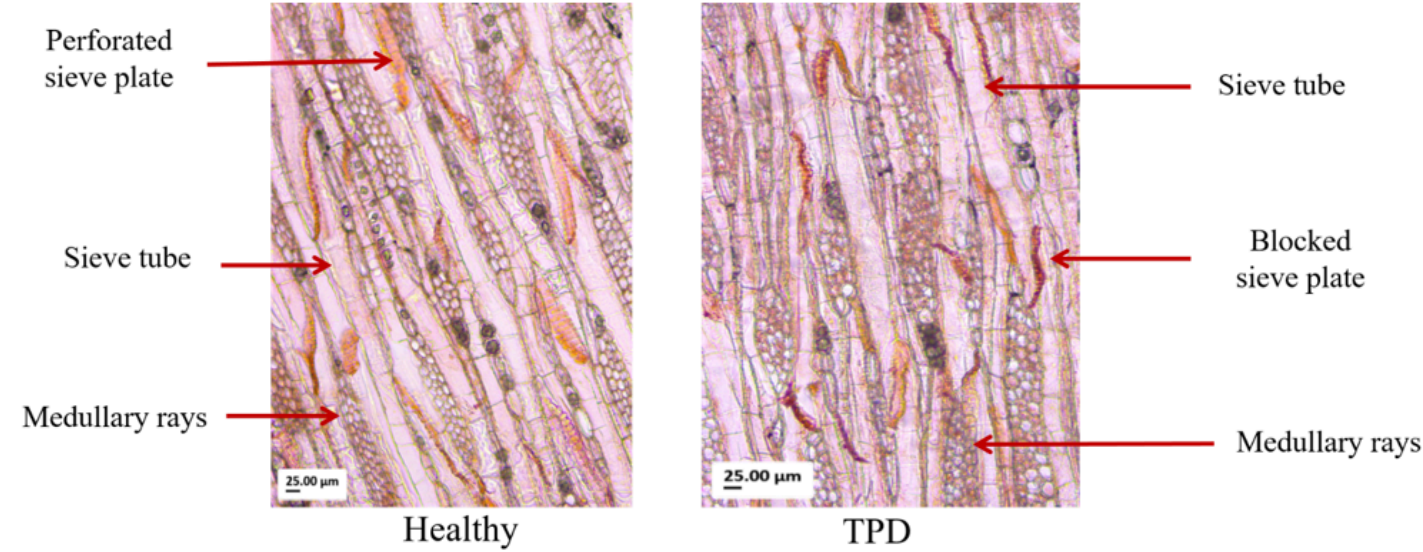


Figure 3

(a) Tangential longitudinal section of inner soft bark region stained with Congo red (10X magnification)

Fig. IV

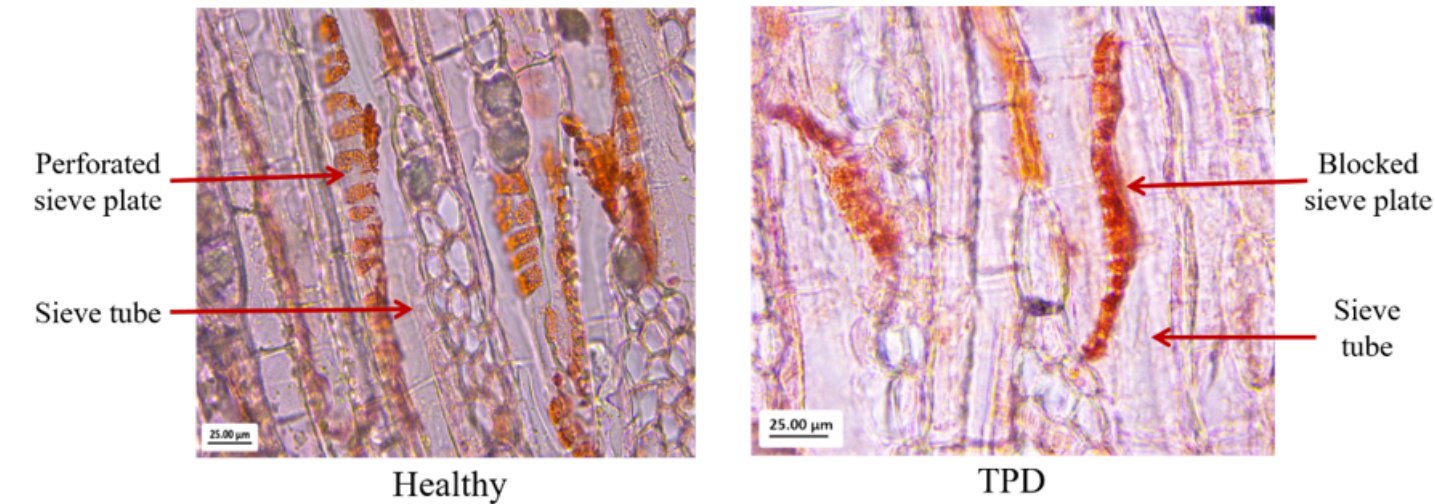


Figure 4

Sieve plates (40X magnification)

Fig. V

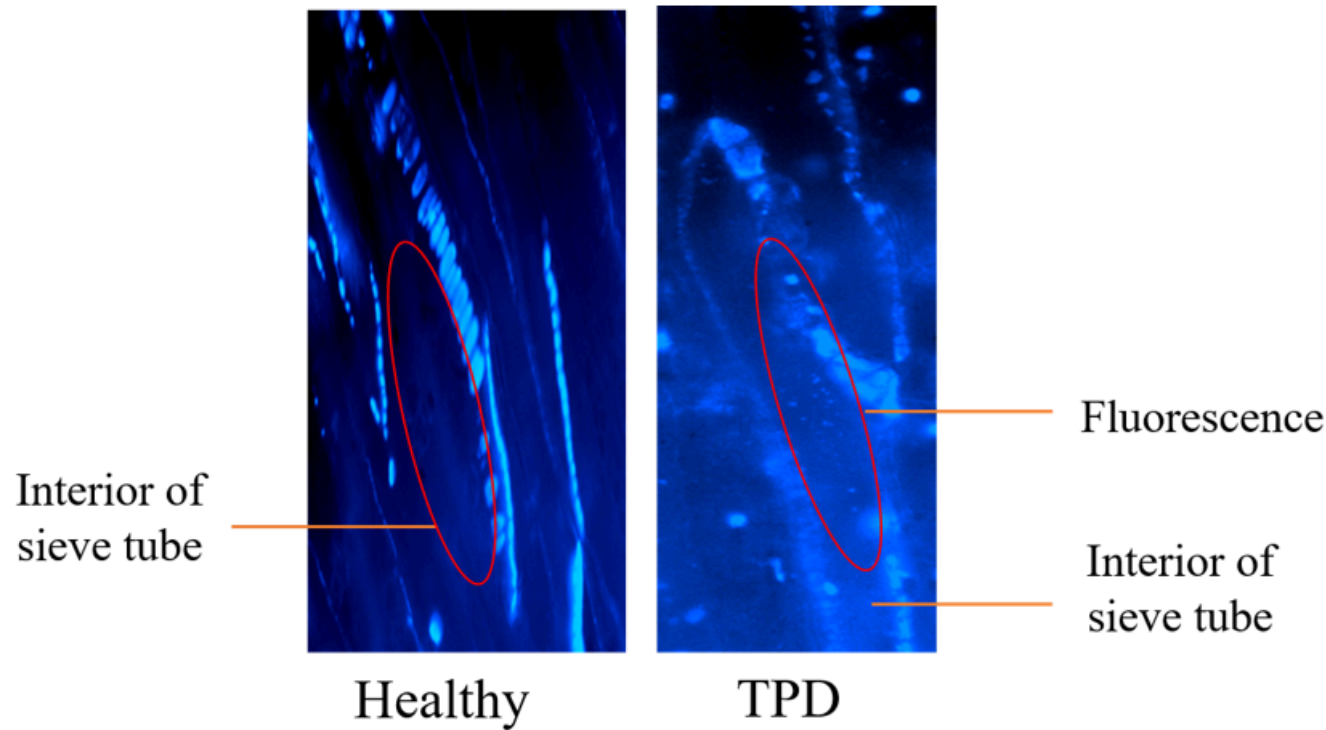


Figure 5

Confocal laser scanning micrograph of sieve tubes

Fig. VI

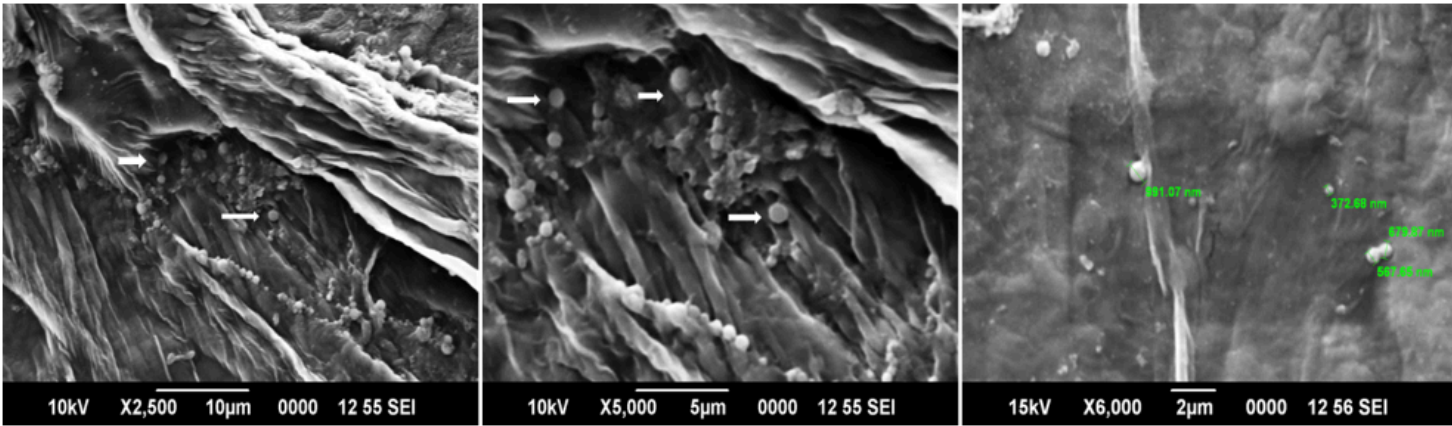


Figure 6

Scanning electron micrograph of sieve tubes of TPD affected bark

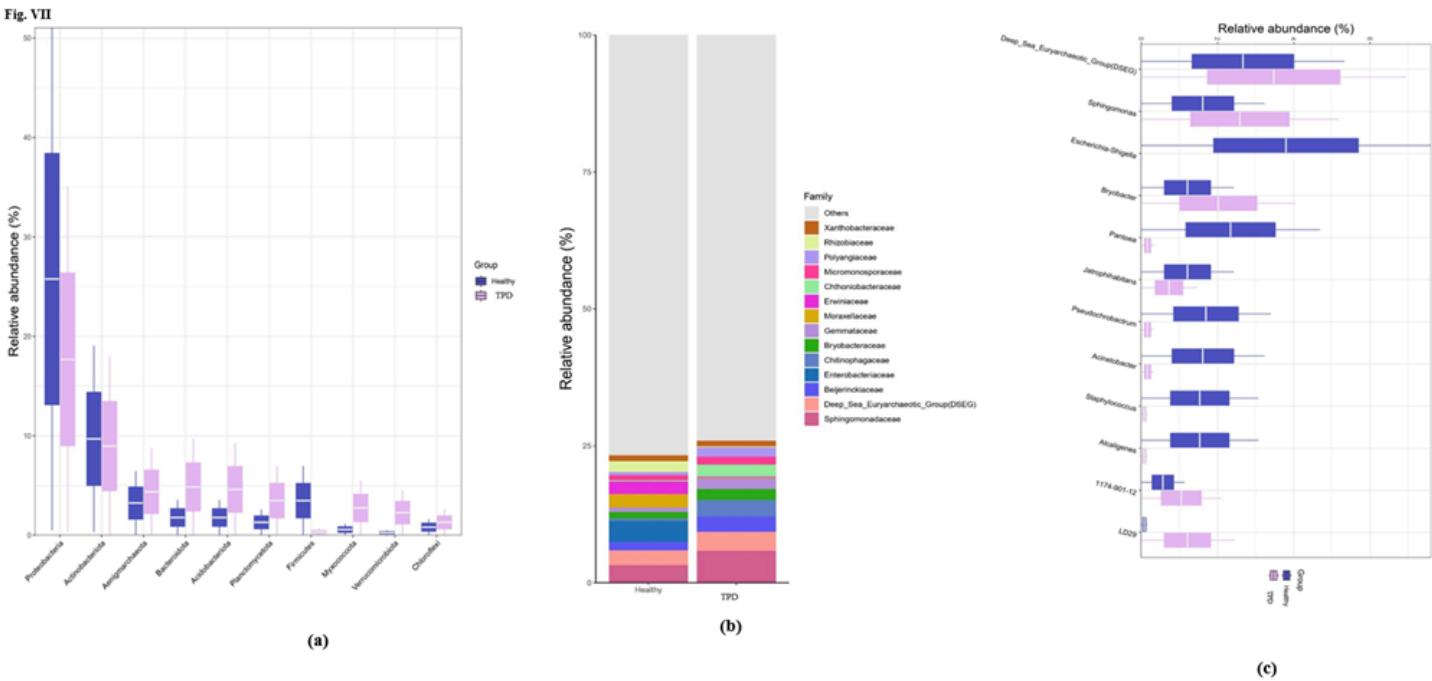


Figure 7

(a) Abundance of microbes at phylum level (b) Abundance of microbes at family level (c) Abundance of microbes at genus level

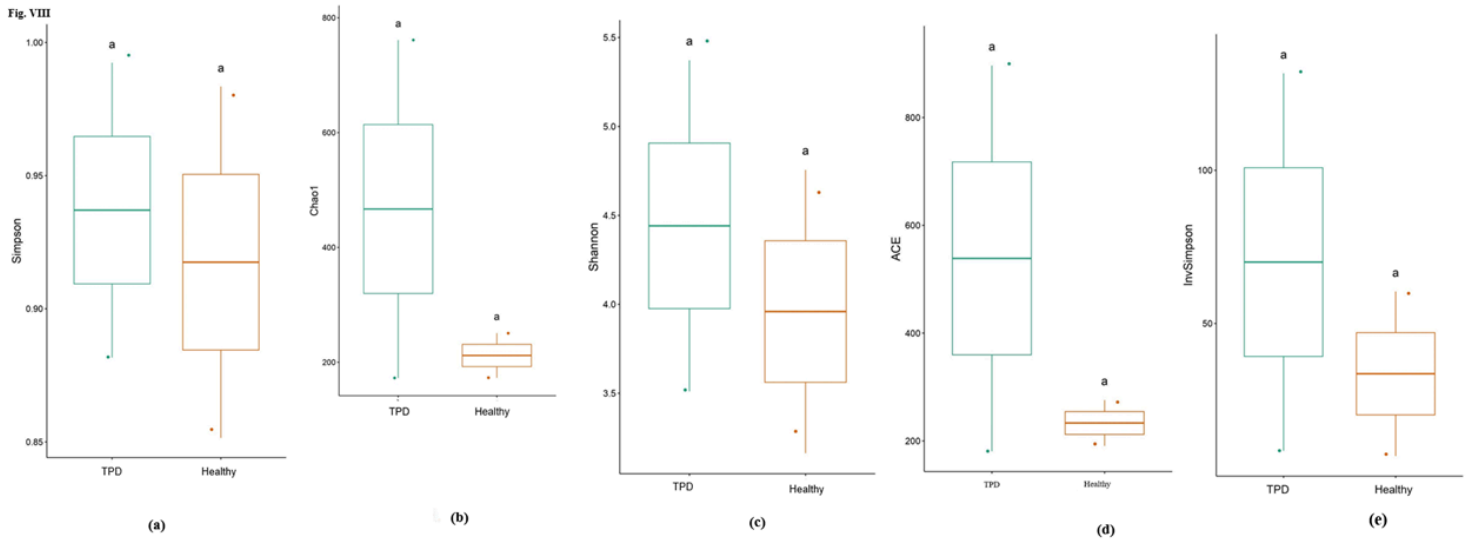


Figure 8

(a) Simpson index (b) Chao index (c) Shannon-Wiener index (d) ACE index (e) Inverse Simpson index

Fig.IX

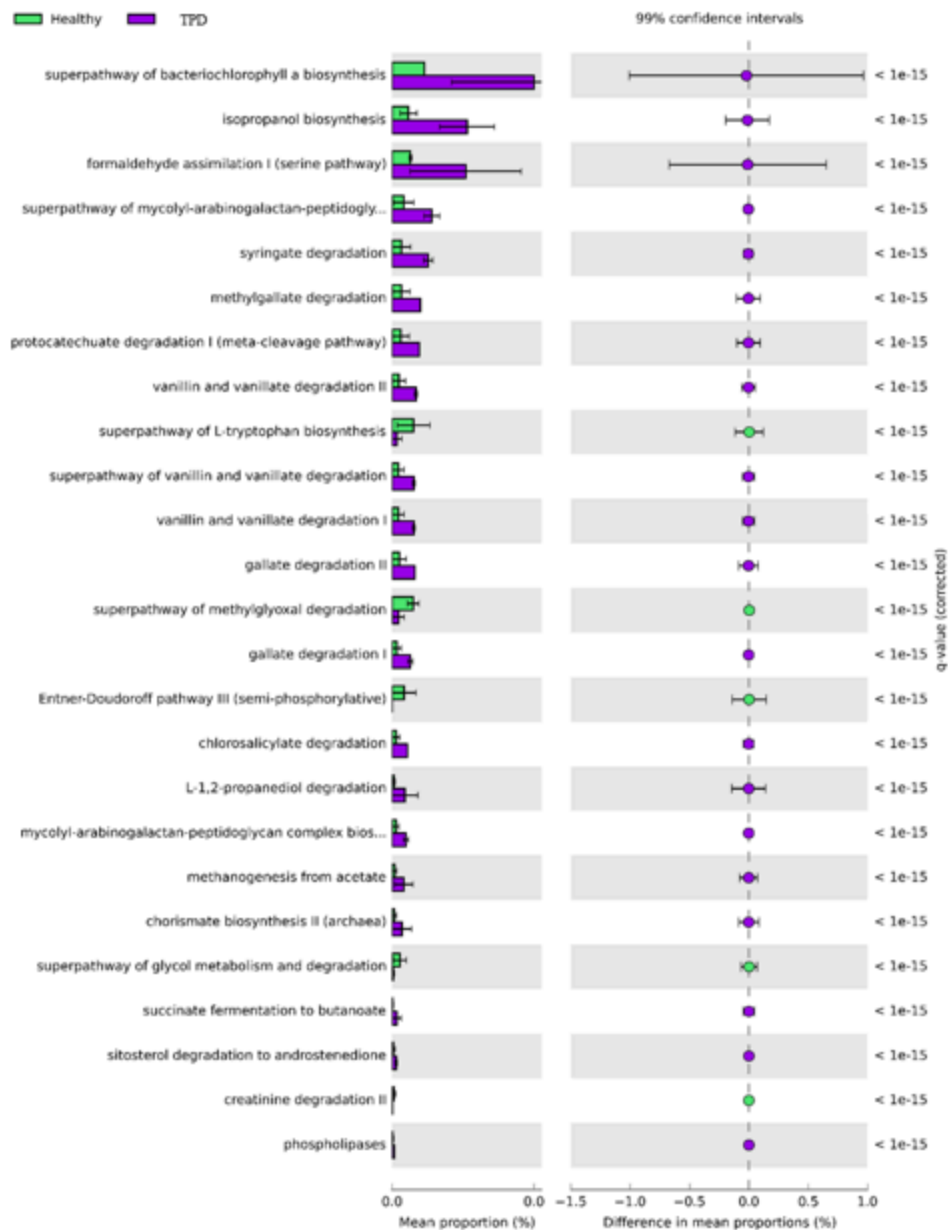


Figure 9

16S rRNA based predicted metabolic pathways