USE OF NEXT GENERATION SEQUENCING FOR CHARACTERIZATION OF MICROBIOMES FROM PHYTOPLASMA / "HUANGLONGBING" INFECTED PLANTS
Bacterial microbiomes in plant and insect materials infected by *'Candidatus* Liberibacter' and *'Candidatus* Phytoplasma' species

■ THE PROBLEM ADDRESSED

Verifying the bacterial populations

Plants are inhabited by a wide diversity of both beneficial and harmful microorganisms such as fungi, bacteria and viruses. These microorganisms, the microbiome, closely interact in networks that can for instance be antagonistic or symbiotic. *'Candidatus* Phytoplasmas' and *'Ca.* Liberibacter' species are part of this network and compete with the remaining microbiome for nutrients and space interacting with other plant-associated microorganisms. This interaction could play important roles for the success of infections and for the ability to spread from plant to plant by insect vectors. Furthermore, the genetic variability of these bacteria, which is often overseen by current methods, could be important for pathogen survival, development of symptoms and transmission by insect vectors. A large number of samples from diverse geographical location, diverse plant and insect species and infected by these pathogenic bacterias confirmed by nested-PCR testing were used for the microbiome determination.

Host species	Country of origin	Partner	Number of samples
Citrus	Guadeloupe	ASSO	15
Citrus	Jamaica	CIB	5
Citrus	Mexico	COLPO	9
Citrus	Cuba	IIFT	24
Citrus	South Africa	PTHSL	8
Insects	Mexico	COLPO	46
Insects	Cuba	IIFT	30
Coconut palm	Jamaica	CIB	19
Coconut palm	Mexico	CICY	14
Coconut palm	Mexico	COLPO	12
Coconut palm	Ghana	CSIR	49
Coconut palm	Cuba	IIFT	6
Grapevines	Chile	UCHIL	14
Grapevines	Italy	UNIBO	84

• Table. List of the samples used in the microbiome study provided by Youri Uneau (ASSO, Guadeloupe), Wayne Myrie (CIB, Jamaica), Ndede Yankey (CSIR, Ghana), Carlos Oropeza (CICY, Mexico), Carlos Fredy Ortiz (COLPO, Mexico), Maritza Luis-Pantoja and Camilo Paredes-Tomás (IIFT, Cuba), Gert Pietersen (PTHSL, South Africa), Gerhard Pietersen (SU, South Africa), Nicola Fiore (UCHIL, Chile).

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■ THE PRACTICE/INNOVATION PROPOSED BY TROPICSAFE

Setting up methods for microbiome characterization

For characterization of *'Ca.* Phytoplasma' and *'Ca.* Liberibacter' associated microbiomes, profiling by DNA sequencing could be an efficient approach. Development and implementation of next-generation sequencing technologies (NGS) have revolutionized methods in microbial ecology by enabling high-resolution community profiling. Methods and primers for the analysis of bacteria already exist and have been intensively used. When possible, use of target-specific primers, that amplify bacterial 16S rDNA sequences while avoiding amplification of plant organelle DNA sequences, is preferred. For these reasons, a set of primers (799F and 1193R) designed for the analysis of plant-associated bacterial communities (Hu *et al.*, 2018), was initially tested. However, phytoplasma DNA is not completely matching to this primer pair and therefore these primer sequences were optimized.

■ HOW IS TROPICSAFE IMPLEMENTING IT?

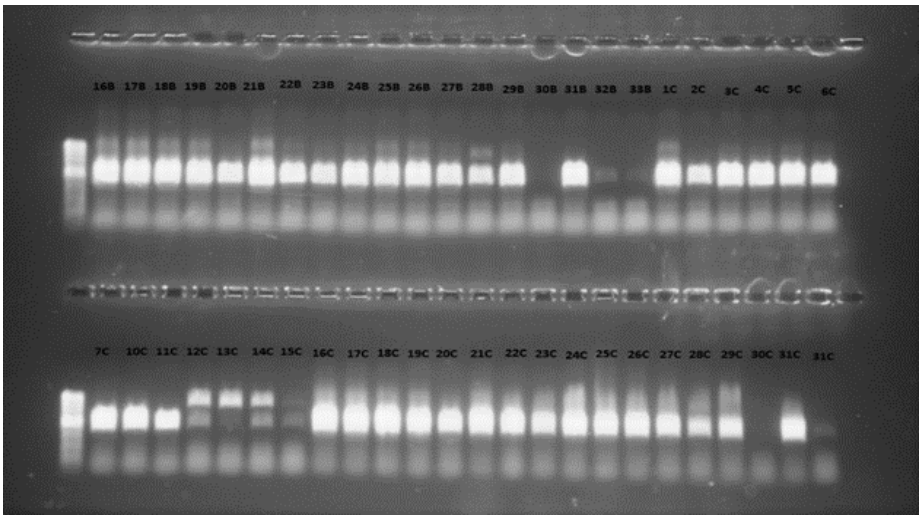
Amplification and sequencing procedures

Variants of the 799F and 1193R primers were optimized based on nucleotide alignments from *'Ca.* Phytoplasma' and *'Ca.* Liberibacter' ribosomal DNA sequences. These optimized primers contain degenerate nucleotides in order to accommodate their sequence variation compared to other bacteria. Nucleotides that are shown below in red are target specific, while the nucleotides in black are specific for the sequencing procedure. Degenerate nucleotides are underlined.

BacF1-799/1193 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACMGGATTAGATACCCKG

BacR1-799/1193 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTNRTCCYCACCTTCC

These primers were tested using DNA from a number of *'Ca.* Phytoplasma' and *'Ca.* Liberibacter' infected host plants with an optimized PCR amplification protocol (94°C for 5 minutes followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes). This resulted in clear DNA bands of the expected size. After optimization, samples primarily originating from Italy, Ghana, Cuba, South Africa, but also from other countries that had been collected by project partners, were characterized by Illumina sequencing and the sequencing reads were computationally and statistically analysed. In total, approximately 400 samples originating from grapevine, palm and citrus together with samples from alternate host plants and insect vectors were analysed.



• Example of amplification using the optimized 799/1193 degenerate primers on a set of DNA samples from different host plants (grapevine and palm). The band of interest is the strong band at approximately 500 nucleotides. At the left side of each panel, a molecular marker is shown.

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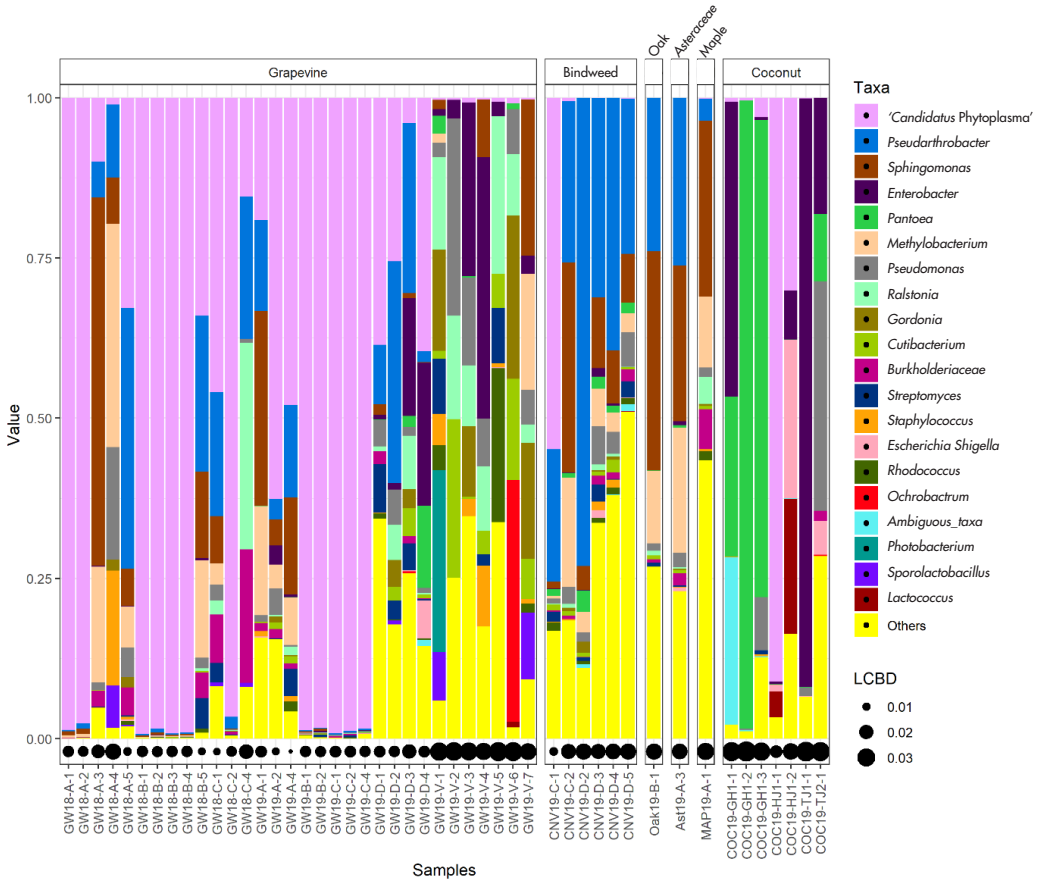
■ HOW IS IT WORKING?

Bacterial differentiation results

The optimized method for characterization of microbial prokaryotic populations in plants infected with *'Ca.* Phytoplasma' and *'Ca.* Liberibacter' successfully amplified target prokaryotic DNA, while no notable amplification of plant organelle DNA was observed after taxonomic assignment of the sequence reads. This shows in the below figure that the proportion of *'Ca.* Phytoplasma' reads (pink bars) of the total microbiome in the individual samples is highly variable. Stacked vertical bars show the distribution of the microbial taxa within each sample in an example of a comparison between samples of palm, grapevine and a range of alternative hosts.

Generally, grapevine samples had a much higher proportion of *'Ca.* Phytoplasma' reads compared to other prokaryotes, and in several cases, phytoplasmas were taking up almost 100% of the sequence reads. Within phytoplasma reads, sequence variation was likewise observed. In the samples from palm, 71 different sequence variants were identified, and of these, 5 sequence variants were identified in several samples. In the samples from grapevine, 82 different sequence variants were detected, and 5 of these were identified in several samples and with notable read numbers in at least more than one sample. Therefore, these sequence variants are considered valid.



• Stacked vertical bars showing the relative abundance of *taxa*, present in each sample, at phylum level from grapevine, bindweed, oak, *Asteraceae*, maple and coconut. *'Ca.* Phytoplasma' reads are shown in pink. The Local Contribution to Beta Diversity (LCBD) is a comparative index of uniqueness with large values indicating the samples that have strongly different species compositions compared to the others. LCBD values are plotted as bubbles under stacked bar plots to indicate samples that differ markedly in their composition.

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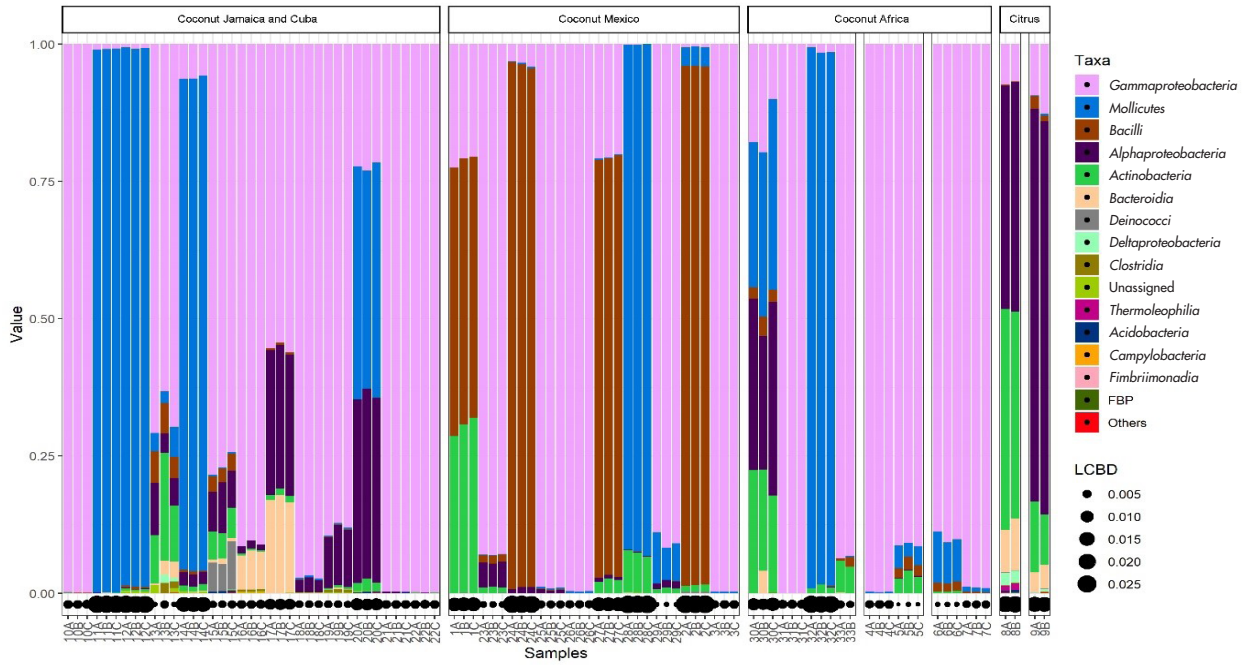

• Stacked vertical bars showing the relative abundance of the most abundant *taxa*, present in each sample. In the citrus samples (last four columns), HLB is within the *Alphaproteobacteria*, while phytoplasma are in the *Mollicutes*. The Local Contribution to Beta Diversity (LCBD) is a comparative index of uniqueness with large values indicating the samples that have strongly different species compositions compared to the other ones. LCBD values are plotted as bubbles under stacked bar plots to indicate samples that differ markedly in their composition.

KEY WORDS

Microbiome, amplification, bacteria, endophytes

FURTHER INFORMATION

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