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Journal:	*FEMS Microbiology Ecology*
Manuscript ID	FEMSEC-20-06-0383.R1
Manuscript Type:	Research article
Date Submitted by the Author:	n/a
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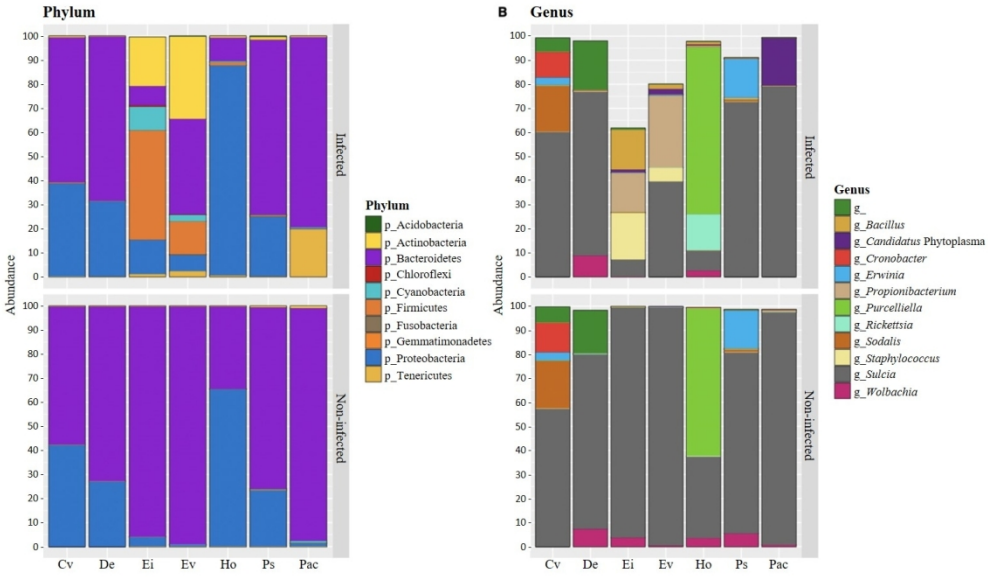
Keywords:	grapevine yellows, Wolbachia, Sulcia, microbial resource management, phloem-limited bacteria



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Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

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One sentence summary: This study describes the microbial community associated with insect vectors of Bois noir disease of grapevine in relation to presence/absence of its etiological agent, '*Candidatus* Phytoplasma solani'.

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ABSTRACT

Bois noir is a grapevine disease causing severe yield loss in vineyards worldwide. It is associated with '*Candidatus* Phytoplasma solani', a phloem-limited bacterium transmitted by polyphagous insects. Due to its complex epidemiology, it is difficult to organize effective containment measures. This study aimed to describe the bacterial microbiota associated with '*Candidatus* Phytoplasma solani' infected and non-infected insect hosts and vectors to investigate if phytoplasma presence can shape the microbiota. Alpha-diversity analysis showed a low microbiota diversity in these insects, in which few genera were highly abundant. Beta-diversity analysis revealed that the xylem- and phloem-feeding behavior influences the microbiota structure. Moreover, it highlighted that phytoplasma infection is associated with a restructuring of microbiota exclusively in Deltocephalinae insect vectors. Obtained data showed that '*Candidatus* Phytoplasma solani' may have adverse effects on the endosymbionts *Sulcia* and *Wolbachia*, suggesting a possible fitness modification in the insects. The phytoplasma-antagonistic *Dyella* was not found in any of the examined insect species. The results indicate an interesting perspective regarding the microbial

signatures associated with xylem- and phloem-feeding insects, and determinants that could be relevant to establish whether an insect species can be a vector or not, opening up new avenues for developing microbial resource management-based approaches.

Keywords: grapevine yellows; *Wolbachia*; *Sulcia*; microbial resource management; phloem-limited bacteria

INTRODUCTION

Diseases that are transmitted by vectors are not only a threat to human health, but can also cause disastrous losses in agriculture, being a threat for livestock and plants upon which we depend for food. Most of the vectors that transmit diseases are arthropods, among which insects and mites can transmit a wide range of pathogens to a broad range of hosts (Ciancio 2016).

Among the plant pathogens that are transmitted by vectors, phytoplasmas deserve a specific mention due to their unique nature, being obligate bacterial pathogens with a broad host range that localize in the phloem of their host plant. However, they have a much stricter specificity when it comes to their insect vectors, as several molecular recognition stages are needed for the phytoplasmas to pass from the insect gut to the hemolymph and ultimately to the salivary glands of the vector, from where they can infect new plants (Namba 2019).

Each phytoplasma can have different vectors but all known vectors are insects belonging to the order Hemiptera, suborder Auchenorrhyncha and Sternorrhyncha, in particular leafhoppers (family Cicadellidae), planthoppers (superfamily Fulgoroidea), and psyllids (superfamily Psylloidea) (Weintraub and Beanland 2006; Alma *et al*. 2015). This study focuses on '*Candidatus* Phytoplasma solani', associated, among others, with grapevine Bois noir, the most widespread disease in the complex of grapevine yellows (Quaglino *et al*., 2013). This complex includes grapevine diseases, associated with genetically and biologically distinct phytoplasma species, that induce common symptoms (desiccation of inflorescences, berry shrivel, leaf discolorations, reduction of growth, and irregular ripening of wood), and cause serious economic damage and yield loss in vineyards (Belli *et al.* 2010; Angelini *et al*. 2018).

The epidemiological cycle associated to Bois noir is extremely complex and was recently discovered to include not only the most well-known vectors *Hyalesthes obsoletus* (Maixner 1994) and *Reptalus panzeri* (Cvrkovic *et al*. 2014), but also other eight species: *Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*,

Laodelphax striatella, *Phylaenus spumarius*, and *Psammotettix alienus*/*confinis* (Quaglino *et al*. 2019).

Since the cycle includes so many highly polyphagous insects and a very broad range of secondary wild hosts, it is difficult to organize effective prevention and containment measures (Bertaccini *et al.* 2014; Moussa *et al*. 2019; Quaglino *et al.* 2019). Moreover, the typical management strategies for phytoplasma diseases, based on the control of the vector with insecticides and the removal of infected plants (Bianco *et al*. 2011), are not effective against '*Ca.* P. solani' (Angelini *et al*. 2018). For this reason, other methods are being envisioned, including the use of Microbial Resource Management (MRM).

MRM is the proper management of the microbial resources available in a given ecosystem in order to solve a practical problem by directing the potential of microorganisms. In particular, on the topic of control of insect vectors, some first steps have already been taken towards defining the composition and functionality of microbial communities associated with insects (Marzorati *et al.* 2006; Miller *et al.* 2006; Crotti *et al.* 2012).

Insects, like all other animals, maintain several symbiotic interactions with their associated microbial community, which has a great influence on their fitness, evolution, and diversity (Margulis and Fester 1991; Ruby *et al*. 2004). The microbial community can contain beneficial symbionts, called mutualists, but also detrimental ones, which are parasites or pathogens, and the dynamic balance found in a microbial community can produce either a positive or negative effect for the health of the host (Berg *et al.* 2014; Lebeis 2014). An MRM approach to control insect vectors would therefore be performed by manipulating their microbial community to promote the effect of naturally present antagonistic microorganisms (Trivedi *et al.* 2016).

A negative prospect for this strategy is that, as the interactions between environment, host, and microbiota are very complex and influenced by several variables (Trivedi *et al.* 2015; Douglas 2015; Fonseca-García *et al.* 2016), more studies need to be conducted in the description of the bacterial community associated to these vectors before its manipulation can become a viable option. The positive prospect, since these phloem-feeding insects rely heavily on obligate bacterial symbionts to provide nutrients which are lacking in their unbalanced diet (Buchner 1965; Baumann 2005; Bourtzis and Miller 2006; Skidmore and Hansen 2017), is that these insects will be particularly susceptible to unbalances in their microbial community.

A main actor in these obligate mutualistic interactions is '*Candidatus* Sulcia muelleri', a bacterial species that greatly reduced its genome as it evolved as an obligate symbiont; moreover, it is documented to be strictly associated to leafhoppers and planthoppers, among other hosts (Moran *et al.* 2005; McCutcheon *et al.* 2009). This bacterial species is involved in the synthesis of several

amino acids necessary for the insect host (McCutcheon and Moran 2007). Other mutualistic bacteria involved in these interactions belong to the genera *Nasuia* and *Sodalis* (Kobiałka *et al*. 2018).

Another bacterial genus interesting for MRM approach is *Wolbachia*, ubiquitous endosymbionts associated with over 60% of known insect species, as well as other arthropods and nematodes (Hosokawa *et al.* 2010; Zug and Hammerstein 2012; Newton and Rice 2020). *Wolbachia* species are cytoplasmically inherited and known as reproductive parasites due to their ability to manipulate reproduction such as sperm-egg incompatibility (cytoplasmic incompatibility), parthenogenesis induction, male killing, and feminization, making it a possible biocontrol agent against the vectors (Werren 1997; Stouthamer *et al.* 1999; Werren *et al.* 2008; Brelsfoard and Dobson 2009; Chuche *et al*. 2017). Nevertheless, several studies showed that *Wolbachia* can act as mutualistic towards insect hosts, modulating nutrition and immune responses (Hosokawa *et al.* 2010; Iturbe-Ormaetxe *et al*. 2011; Newton and Rice 2020). Moreover, recent studies proposed that *Wolbachia* can act as biocontrol agent of insect-transmitted pathogens, including phytoplasmas, by increasing latency period and blocking pathogen transmission (Shaw *et al.* 2016; Chuche *et al*. 2017).

Dyella-like bacterium (DLB), gram-negative, aerobic, rod-shaped endophytic bacteria belonging to the family Rhodanobacteraceae, can be acquired by feeding and has shown a potential biocontrol activity against phytoplasmas and their cultivable relative *Spiroplasma melliferum* (Iasur-Kruh *et al.* 2017, 2018). The possible mechanisms of DLB antagonism towards phytoplasmas have been hypothesized to be (i) competition for nutrients or colonization niches, (ii) induction of plant systemic resistance, (iii) secretion of plant growth hormones, or (iv) secretion of phytoplasma growth inhibitory substances (Eljounaidi *et al.* 2016).

In this scenario, the current study aims to characterize through an Next Generation Sequencing (NGS) approach the bacterial community associated with selected '*Ca*. P. solani' insect hosts, both infected and non-infected by the phytoplasma, with the following goals: (i) describe the bacterial communities in different insect hosts of '*Ca*. P. solani'; (ii) determine whether the presence of '*Ca*. P. solani' affects the bacterial community, in particular if it can cause a dysbiosis (also called dysbacteriosis) or increase diversity; (iii) evaluate the presence of possible antagonists towards the insect (e.g. *Wolbachia* spp.) or phytoplasma (e.g. *Wolbachia* spp. and *Dyella*-like bacteria); (iv) investigate the effect of '*Ca*. P. solani' on the obligate endosymbiont '*Ca.* Sulcia' spp.. The selected insects are the main vector *H. obsoletus*, newly reported vectors (phloem-feeders: *A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, and *P. alienus*/*confinis*; xylem-feeder: *P. spumarius*), and *Cicadella viridis*, one of the most abundant

insects living in Italian vineyard, harboring with high infection rate but not vectoring '*Ca*. P. solani' (Quaglino *et al*. 2019), and characterized by xylem-feeding activity. *C. viridis* was included in the study for comparing the microbiota associated with xylem- and phloem-feeders, and investigating the phytoplasma influence on the microbiota structure in comparison with vectors. *R. panzeri* was not among the selected vectors because it is not found in the studied area.

Achieving the previously mentioned aims regarding the description of the bacterial communities may help in devising MRM-based approaches to achieve the main objective of biological control of '*Ca*. P. solani' and its insect vectors.

MATERIALS AND METHODS

Insect collection

Specimens of the insect species *A. makarovi*, *C. viridis*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius*, and *P. alienus*/*confinis* were captured by sweep entomological net in mid-July 2018 in the Chardonnay organic vineyard (Franciacorta, Lombardy Region, North Italy; N 45°35′38.12″, E 10°09′34.32″) where new insect vectors of '*Ca*. P. solani' had previously been identified (Quaglino *et al*. 2019). Insect individuals were stored in ethanol 90%, transferred to the lab for species identity confirmation by stereomicroscope based on the taxonomic keys of den Bieman *et al.* (2011), and maintained in absolute ethanol at 4°C till use. Regarding the genus *Psammotettix*, given that the dichotomous keys are related only to males, the species *P. alienus* and *P. confinis* were considered together.

Total nucleic acids extraction and suitability for amplification

Total nucleic acids (TNAs) were extracted from ethanol preserved insects (dried by filter paper) through homogenization in a CTAB-based buffer [2% w/v cetyltrimethylammonium-bromide (CTAB); 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HCl pH 8.0; 0.5% ascorbic acid]. After incubation at 60°C for 20 min, TNAs were separated with one volume of chloroform: isoamyl alcohol 24:1 v/v solution and precipitated with the addition of one volume of cold isopropanol. The TNAs pellet was then washed with ethanol 70%, air dried, dissolved in 30µL of TE buffer pH 8.0, and maintained at −20 °C until use (Moussa *et al.* 2019).

The suitability of the extracted TNAs for amplification was tested through a bacterial *16S rRNA* gene PCR assay using the universal primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') / 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). PCR reactions were conducted in Applied Biosystems 2720 thermocycler (Applied Biosystems, Monza, Milan) with the

following conditions: 2 min at 95 °C; 35 cycles consisting of 1 min at 95 °C, 1 min 30 s at 50 °C and 2 min at 72 °C; 10 min at 72 °C. PCR reactions were performed in 25 µL volume containing 50 µM of each dNTP, 0.4 µM of each primer, 1.5 mM MgCl2, 1× polymerase buffer, 1 unit GoTaq polymerase enzyme (Promega, Milan, Italy). PCR mixture devoid of TNAs was employed as negative control. PCR products were analyzed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator. Only the samples that gave positive amplification with this reaction were considered for further analyses.

Molecular detection of '*Candidatus* Phytoplasma solani'

The presence of '*Ca*. P. solani' in collected insects was verified by species-specific nested PCR-based amplification of the *stamp* gene using the primer pair *Stamp*-F (5'-GTAGGTTTTGGATGTTTTAAG-3') / *Stamp*-R0 (5'-AAATAAAAGAACAAGTATAGACGA-3'), followed by the primer pair *Stamp*-F1 (5'-TTCTTTAAACACACCAAGAC-3') / *Stamp*-R1 (5'-AAGCCAGAATTTAATCTAGC-3') (Fabre *et al*. 2011). PCR reactions were conducted in Applied Biosystems 2720 thermocycler with the following conditions: 4 min at 94 °C; 35 cycles consisting of 30 s at 94 °C, 30 s at 56 °C (direct PCR) or 52 °C (nested PCR) and 1 min 30 s at 72 °C; 7 min at 72 °C. PCR mixture devoid of TNAs was employed as negative control. PCR reaction mixtures and PCR products visualization were as described above for bacterial *16S rRNA* gene.

Illumina Mi Seq sequencing

Based on the molecular detection of '*Ca*. P. solani' and the requested TNAs quantity (at least 0.5 µg) / quality (ratio 260/280 nm ~2), TNAs extracted from 96 insect specimens were selected to undergo Illumina Mi Seq sequencing. These 96 samples were picked to ensure that at least five samples for each insect species were included in both the '*Ca*. P. solani'-infected and non-infected groups.

Next generation sequencing library preparations and Illumina Mi Seq sequencing were conducted by an external provider (Personal Genomics, Verona, Italy). The bacterial *16S rRNA* gene hypervariable region V4 libraries were prepared using the forward primer 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3'), and the amplification of sequences belonging to mitochondria was blocked using a PNA blocker (Lundberg *et al*. 2013). Metagenomic sequencing was performed using the Illumina Miseq 300PE sequencing technology. Obtained reads were deposited in the EMBL-ENA under the project number PRJEB38750.

Processing of high-throughput sequencing data

The raw sequencing reads were initially filtered, to remove low quality sequences, trim primers and Illumina adapters. The initial quality control of the reads was performed with FastQC v0.11.5. Primers were trimmed with the cutadapt tool version 1.14 (Martin 2011) while adapters were trimmed with Sickle version 1.33 (https://github.com/najoshi/sickle) and Scythe version 0.991 (https://github.com/vsbuffalo/scythe). The obtained reads were analyzed using the QIIME 2 pipeline (Bolyen *et al.* 2019) in order to assign them to OTUs. Allocation to OTUs and clustering were performed using uclust with a minimum similarity of 97% (default). Identified OTUs from representative sequences were aligned to Green-genes (http://greengenes.lbl.gov/) using R-studio. Chloroplast and mitochondria (these constituted only 1-2% in some samples) were filtered as well as rare OTUs (i.e., singletons and OTUs < 10). The resulting OTU table was then used for the subsequent analyses.

Diversity and statistical analysis

After quality filtering and rarifying to 1600 sequences per sample, Alpha-diversity indices (Shannon index, ChaoI and observed OTU) were calculated to ensure that enough sequencing coverage had been achieved by using BiocManager package implemented in the R software (R Project 3.0.2; http://cran.rproject.org/). Observed, Chao1 (Chao 1984) and Shannon H' index (Shannon 1948) were considered for the aforementioned features. Alpha diversity indices were compared between different insect species groups ('*Ca*. P. solani' infected or non-infected). Shapiro test was performed for data normality followed by ANOVA in the case of Observed richness whereas Kruskal test was used for Chao1 and Shanon H' index. Welch t-test was carried out to compare between the infected and non-infected groups of individual species. Beta diversity was assessed by Bray-Curtis (Bray and Curtis 1957) distance matrices and visualized by principal coordinate analysis (PCoA). The PERMANOVA statistical analysis was performed to determine the significance of microbial community differences among the different insect species and infection status with controlled 10^5 permutations. Taxonomic abundance data was calculated using the percentage abundance of OTUs present in the core microbiota. Heat tree was used to plot all the OTUs present in the dataset using the 'metacoder' package. Taxonomic data were plotted using heat trees in which the size and color of tree parts correspond to reads for each taxon as the size of each taxon.

RESULTS

Insects collected and '*Ca*. P. solani' infection rate. A total of 400 individuals were captured. The most abundant species were *E. variegatus* (75 individuals), *P. alienus*/*confinis* (71) and *E. incisus* (59), while *L. striatella* (16), *D. hamata* (8) and *A. makarovi* (6) were scarcely present (Table 1). Bacterial 16S rDNA fragment 27F/1492R was amplified by the TNAs extracted from all insect specimens and not in the negative control, evidencing the TNAs suitability for further molecular analyses. PCR-based amplification of *stamp* gene identified the presence of '*Ca*. P. solani' in 127 out of 400 individuals. Infection rate was >40% in *H. obsoletus*, *E. variegatus*, and *P. spumarius*, >30% in *D. europaea*, *C. viridis* and *E. incisus*, and >10% in *P. alienus*/*confinis*. The phytoplasma was not identified in the least abundant species *L. striatella*, *D. hamata* and *A. makarovi* (Table 1); these latter three species were thus not included in microbiota analyses. For each of the other seven insect species, the number of '*Ca*. P. solani'-infected and -non-infected specimens selected for microbiota analyses is reported in Table 1.

Bacterial diversity analysis

Poor quality sequences were obtained in twelve out of 96 insect specimens that were excluded from further analyses (Table 1). Sequencing of the V4 region of the *16S rRNA* gene on the '*Ca.* P. solani' infected and non-infected group produced, after filtering out organellar sequences and rare OTUs, a total of 527466 sequences belonging to 363 different OTUs. Out of all the obtained sequences, 228190 belong to '*Ca.* P.solani' infected group and 299276 to the non-infected group. Number of sequences and OTUs obtained from the '*Ca*. P. solani' infected and non-infected group are reported in Table 2.

The alpha diversity indices of Observed, Chao1 and Shannon were used for this study as shown in Fig. 1. The observed OTUs were considered to show the absolute richness. The values of this parameter range from a minimum average of 17, found in non-infected *E. incisus* and *D. europaea*, to a maximum of 106, found in infected *E. incisus*. The corrected estimation of richness made through the Chao1 index are very close to the value of Observed for most samples, indicating that the sequencing has reached an adequate depth, having very few singletons and a low number of estimated undetected OTUs. The Shannon index, indicating the evenness of species distribution ranges from a minimum of 0.089 in non-infected *E. variegatus*, to a maximum of 2.25 in infected *E. incisus*. For *E. incisus, E. variegatus* and *H. obsoletus*, the number of Observed OTU and the Shannon index are significantly different between infected and non-infected samples, indicating that the presence of the phytoplasma has a strong effect on the alpha-diversity of the bacterial community in these species. For all other considered insect species, no statistically significant difference was found between these values for infected and non-infected groups. The bacterial

distribution of the different insect species both infected and non-infected groups were characterized in terms of the relative taxonomic abundance. A total of 18 phyla, 46 classes, 58 orders, 89 families, 100 genera and 35 species (of which a total of 277 with an unidentified taxa).

Core microbiome

In order to highlight the existence of an identifiable common core microbiome, the group of members shared among the microbial community of the infected and non-infected groups of the different insect species were identified. The bacterial communities in these insect populations are clearly distinct and do not share a common core as no single OTU is shared (i) among individuals of all insect species regardless of infection, (ii) among infected individuals regardless of insect species, (iii) among non-infected individuals regardless of insect species. Venn diagrams were used to represent the number of OTUs found exclusively in the infected group, non-infected group, or shared between the two groups (Fig. 2). For most of the analyzed species, a common trend can be identified with infected individuals showing a much higher number of unique OTUs compared to non-infected samples. This difference is particularly pronounced in *E. incisus* and *E. variegatus* (Fig. 2 and 3). This is true for all species, except *D. europaea* and *P. spumarius*, for which the number of unique OTUs in infected and non-infected samples is very similar (Fig. 2). Interestingly, regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65 shared OTUs (Fig. 2).

Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between infected and non-infected in all species. Other relevant bacterial genera that are core between infected and non-infected in particular species are *Cronobacter* and *Sodalis* (*C. viridis*), *Erwinia* (*P. spumarius*), *Propionibacterium* (*E. variegatus*), *Purcelliella* (*H. obsoletus*), and *Rickettsia* (*H. obsoletus*).

Bacterial community structure

Venn diagram representation showed a qualitative difference among OTUs identified in infected and non-infected individuals in the species, without considering the vital quantitative aspect in describing the community structure. To compare the microbial community structure among the '*Ca.* P. solani' infected and non-infected individuals within and among insect species, principle coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 4).

The graph shows that the species is a major driver of diversity among the microbial communities, as each species tends to form a separate cluster. From this analysis, two groups of insects can be identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on species alone, with the single samples of infected and non-infected insects overlapping and mixing with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct clusters based on species for non-infected samples, but the infected samples do form clusters based on species, distinct from the non-infected samples within the same species. These results were confirmed by an Adonis multivariate analysis of variance, showing that there are statistically significant differences between the structure of the community in infected and non-infected samples of *E. incisius* (p=0.001), *E. variegatus* (p=0.013) and *P. alienus/confinis* (p=0.006), while no significant differences were found in the other four species.

Bacterial abundance and distribution

The composition in taxa of the microbial communities according to the different insect species as well as the different infection status are reported in the bar plots in Fig. 5. All detected OTUs could be assigned to one of ten phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Proteobacteria, or Tenericutes (Fig. 5A). In most analyzed samples, the most abundant phylum is Bacteroidetes, which can compose up to 99% of the total community, as for the non-infected *E. variegatus*. This dominance of Bacteroidetes is seen in all non-infected samples, except for *H. obsoletus*, and also in some infected insect species: *D. europaea*, *P. spumarius*, and *P. alienus/confinis*. The second most abundant phylum in most samples is Proteobacteria: this phylum is the most abundant in *H. obsoletus*, both infected and non-infected, and is also highly abundant also in *C. viridis*, *D. europaea*, and *P. spumarius*, both in infected and non-infected samples. While mostly absent in non-infected samples, the phyla Actinobacteria and Firmicutes are found with higher abundance in the infected samples of *E. incisus*, and *E. variegatus*. Bacteria belonging to the phylum Cyanobacteria are found only in the infected samples of *E. incisus* and *E. variegatus*.

In most of the examined insect species, the microbial community was composed of members of few genera, but at very high abundance. In fact, as it can be seen by comparison of Fig. 5A and 5B, almost the entire abundance of the Bacteroidetes phylum can be ascribed to the genus *Sulcia* alone. Likewise, the Tenericutes abundance is due uniquely to the presence of OTUs of '*Ca.* Phytoplasma' and, in *H. obsoletus*, the abundance of Proteobacteria overlaps with the abundance of *Purcelliella*. In contrast, the microbiota of *E. incisus* shows many more genera, but the 12 most abundant ones only cover 60% of the abundance, while the rest are less abundant genera. Regarding

'*Ca*. Phytoplasma' OTUs, they are found exclusively in infected individuals of all species, but with high abundance (>1%) only in *E. incisus*, *E. variegatus*, and *P. alienus/confinis* (Table 3).

Comparing the infected and non-infected abundance of different genera, it emerges that for some species there are no changes, or very little changes, in the structure of the bacterial community in the presence of absence of '*Ca.* Phytoplasma solani': *C. viridis*, *D. europaea*, and *P. spumarius* (Fig. 5B). For the main host, *H. obsoletus*, the community itself does not seem to undergo great variations in quality, with the addition of only *Rickettsia* in infected samples, but the relative abundance of the members of the community are vastly different. Similarly, for *P. alienus/confinis* the community only shows the addition of '*Ca*. Phytoplasma' between healthy and infected samples, but the abundance of OTUs belonging to this genus is very high, suggesting a strong interaction between this plant pathogen, the host, and the microbial community already present in the host. For the remaining examined species (*E. incisus*, *E. variegatus*) the infection by the phytoplasma is accompanied by a radical change in the microbial community (Fig. 5B).

Regarding the bacterial genera that were of particular interest in this study, it can be seen that (i) the '*Ca.* Phytoplasma'-antagonistic *Dyella* is not found in any of the examined insect species. (ii) *Wolbachia* is found in non-infected specimens of all vector species, but with high abundance (>1%) only in *D. europaea*, *E. incisus*, *H. obsoletus*, and *P. spumarius*; in all examined vector species, with the exception of *D. europaea*, the abundance of this genus is reduced in the infected samples, to the degree of disappearing entirely from the community for *E. incisus* and *E. variegatus*. *Wolbachia* is not found in *C. viridis* regardless of phytoplasma infection (Table 3). (iii) The mutualistic symbiont *Sulcia* makes up for the majority of the microbiota in non-infected specimens of all insects, except *H. obsoletus*. Within phloem-feeders, it showed an abundance >95% in *E. incisus*, *E. variegatus* and *P. alienus/confinis*, ~75% in *D. europaea*, and ~30% in *H. obsoletus*. Within xylem-feeders (*C. viridis* and *P. spumarius*), it showed an abundance <75%. With the exception of the xylem-feeders and *D. europaea*, its abundance is greatly reduced in infected samples, compared to non-infected samples of the same species (Table 3).

DISCUSSION

The insect survey and molecular identification of '*Ca*. P. solani', conducted in this study, confirmed the presence of abundant populations and the unusually high infection rate (>10%) in 2018 for the main vector *H. obsoletus* and for a majority of the insect species recently reported as vectors (Quaglino *et al.* 2019). If the scenario of containing Bois noir disease in vineyards was already bleak due to the high polyphagia of the established insect vectors, the addition of several more vectors is leading to the idea that there are no options to implement any traditional containment

strategy against this disease, its pathogen, or vectors. A comprehensive and thorough investigation of the bacterial diversity in '*Ca*. P. solani' insect vectors is essential for understanding how this pathogen interacts with its hosts and their microbiota, possibly leading to the development of effective prevention and treatment strategies based on the management of the bacterial community in the vectors.

This study analyzes the bacterial community present in insects associated to '*Ca.* P. solani' collected in vineyards in northern Italy, including the main vector *H. obsoletus*, five newly reported vector species (*D. europaea, E. incisus*, *E. variegatus*, *P. spumarius*, and *P. alienus*/*confinis*) and a species that is known to host the phytoplasma but not to transmit it, *C. viridis*. In addition to investigating and describing the bacterial community found in these insects, both when they're infected with '*Ca*. P. solani' and when they aren't, the study focuses on the presence of specific genera of bacteria that have been reported as potentially essential for the survival of the insect (genus *Sulcia*), as potential parasites of the vectors (genus *Wolbachia*), or as antagonistic towards the phytoplasma (genus *Wolbachia* and *Dyella*).

In comparison with previous studies on the topic of the bacterial communities associated to insect vectors of '*Ca.* P. solani', this study uses a more modern technique than those previously employed [LH-PCR, DGGE (Gonella *et al*. 2011); sequencing with Roche 454 (Iasur-Kruh *et al*. 2017)] and extends the range of investigation to more vectors, instead of analyzing just *H. obsoletus*.

Starting from the parameters of alpha-diversity, it is found that in these insects the microbial communities do not have a high diversity, showing a low number of different OTUs that dominate the whole community. This is particularly true for the non-infected samples that showed less than 20 different OTUs for most of the analyzed species. This result is in accordance with what was previously presented regarding the bacterial communities of phloem-/xylem-feeding insects, and it is hypothesized that this is due to their extremely specialized diet which (i) requires specific metabolic processes to implement the insect's own and ensure survival and (ii) comes from a compartment of the plant that is colonized only by very specialized bacteria and therefore acts as a low-diversity reservoir from which the insects ingest bacteria (Colman *et al.* 2012; Jing *et al.* 2014; Overholt *et al.* 2015).

For most species there is no difference in the alpha-diversity parameters between '*Ca.* P. solani' infected and non-infected specimens, indicating that the presence of the pathogen does not lead to a major change in the qualitative composition of the community. Still, for *E. incisus* and *E. variegatus* a statistically significant increase was observed for all parameters in the infected specimens, compared to the non-infected.

The analysis of abundance of the different taxa in the insect species in general revealed microbial communities with low diversity, in which only a handful of genera were present with high abundance: *Bacillus* (Firmicutes), '*Candidatus* Phytoplasma' (Tenericutes), *Cronobacter* (Proteobacteria), *Erwinia* (Proteobacteria), *Propionibacterium* (Actinobacteria), *Purcelliella* (Proteobacteria), *Rickettsia* (Proteobacteria), *Sodalis* (Proteobacteria), *Staphylococcus* (Firmicutes), *Sulcia* (Bacteroidetes), and *Wolbachia* (Proteobacteria).

The results obtained on the description of the bacterial microbiota of *E. incisus* and *P. alienus/confinis* agree with what was previously reported by Kobialka *et al.* (2018), who indicated a microbial community dominated by *Sulcia* for these species.

Regarding *H. obsoletus*, our results that highlighted the presence of the genera *Sulcia*, *Wolbachia*, and *Purcelliella* confirming data previously obtained by Bressan *et al.* (2009) and Gonella *et al.* (2011) in northern Italy, but not those obtained by Iasur-Kruh *et al.* (2017) in Israel. This latter study determined, using both classical and molecular microbiology methods, that the genus *Sulcia* was the most abundant in *H. obsoletus*, followed by *Pectobacterium*. These differences may be explained by several variables, such as the different techniques used, and the different geographical areas from which specimens were sampled, which leads to different climatic conditions and insect diet.

The results obtained on *C. viridis*, with a high abundance of the genera *Sulcia* and *Sodalis* are in accordance to those previously reported by Michalik *et al.* (2014). Intriguingly, these results also revealed that *C. viridis* has a unique microbiota compared to the other insect species analyzed: it has a more diverse composition, in which five different genera have a relevant level of abundance and it's the only species in which we find a high abundance of the genera *Cronobacter* and *Sodalis*. These results suggest that either the higher diversity, leading to a more resilient bacterial community, or these specific genera of bacteria could play a role in determining the non-vector status of this insect. Further studies will be conducted to determine if these elements can indeed be important and relevant for the development of an MRM strategy to reduce the spread of Bois noir.

The results regarding the beta-diversity in each analyzed insect species, infected and non-infected, highlighted the presence of two different groups among the insect species: (i) insects for which the presence or absence of the phytoplasma did not cause a major restructuring of the bacterial community, including the species *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius*; and (ii) insects for which the presence of the phytoplasma, not related to its abundance, caused a major change in the bacterial community, including the species *E. incisus*, *E. variegatus*, and *P. alienus/confinis*. Interestingly, among the analyzed species, these three are the only ones belonging to the subfamily Deltocephalinae. The microbiota associated with members of this subfamily is

usually characterized by the presence of two ancient mutualistic endosymbiotic bacterial genera: *Sulcia* and *Nasuia* (Kobiałka *et al*. 2018). However, several studies reported that the symbiotic systems of Deltocephalinae leafhoppers can be very diverse, driven by processes of symbiont acquisition and replacement, which can include both bacteria and fungi (Nishino *et al*. 2016; Brentassi *et al*. 2017; Kobiałka *et al*. 2018; Mao and Bennett 2020). In our datasets, no OTUs assigned to the genus *Nasuia* were detected. This result is not in accordance with what is reported by Kobiałka *et al*. (2018), which found *Nasuia* in *E. incisus* and *P. alienus*/*confinis*. On the other hand, a similar situation, in which *Nasuia* was not detected and *Sulcia* represented more than 95% of microbiota OTUs, was reported in *Dalbulus maidis* (subfamily Deltocephalinae) (Brentassi *et al*. 2017). *D. maidis* is the vector of '*Ca.* Phytoplasma asteris' (Raygoza and Nault 1998), associated with maize bushy stunt disease, a phytoplasma strictly related to '*Ca*. P. solani' (Quaglino *et al*. 2013). Considering these data, it is reasonable to propose that the symbiotic systems in our insect populations are prevalently based on *Sulcia*. Furthermore, in North Italian vineyards, *Nasuia* was not identified in *Scaphoideus titanus* (subfamily Deltocephalinae), the insect vector of the phytoplasma associated with flavescence dorée disease of grapevine (Sacchi *et al*. 2008). This could suggest the hypothesis that, in North Italy, the environmental conditions of vineyard agroecosystems do not favor *Nasuia* as mutualistic endosymbiont of phytoplasma insect vectors. For the species *C. viridis*, *D. europaea*, *H. obsoletus* and *P. spumarius*, our results are in accordance with what was reported by Fagen *et al.* (2012) regarding the bacterial community of *Diaphorina citri*, the vector of another obligate plant pathogen '*Ca.* Liberibacter asiaticus': the microbiota of these insects was dominated by the same three or four genera regardless of the presence or abundance of the plant pathogen. As the presence of the phytoplasma does affect the microbial community in the other three analyzed species, it becomes evident that it is not the presence of phytoplasma that determines a change in the microbial community, but the interaction between phytoplasma, insect host, and bacterial community. As expected from their common feeding behavior, the xylem-feeding species *C. viridis* and *P. spumarius* showed a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. However, the aforementioned unicity of *C. viridis* microbiota is not due exclusively by its source diet, which is shared by *P. spumarius*, reinforcing the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Regarding the specific genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), interesting considerations can be made for *Sulcia* and *Wolbachia*, while *Dyella* was not found to be present in any of the analyzed specimens. This might be due to the time of sampling, as it was

reported that the presence of *Dyella*-like bacteria increases in the late stage of the season (Iasur-Kruh *et al.* 2017).

In terms of abundance, the genus *Sulcia* was found to be the most abundant in all non-infected insect species except *H. obsoletus* where it was the second most abundant after *Purcelliella*. This result is in agreement with Moran *et al.* (2005) who showed that several Auchenorrhyncha insect lineages, including Cicadomorpha and Fulgomorpha, house a single phylotype bacterium called '*Candidatus* Sulcia muelleri'. In the infected groups there was a dramatic decrease in the genus *Sulcia*; except in the case of *C. viridis*, *D. europaea*, and *P. spumarius* where the reduction was quite low. This reduction in the abundance of *Sulcia* has several possible explanations: the first is that the interaction between the phytoplasma and other members of the microbiota lead to a rise of secondary mutualists, disadvantaging the primary mutualists such as *Sulcia* (Heddi *et al.* 1998); a second hypothesis is related to the host's immune response: it was demonstrated by Galetto *et al.* (2018) that the insect, *E. variegatus* in that study, can activate a strong immune response when interacting with a phytoplasma that is not the one that it usually transmits. This immune reaction could change the bacterial community inside the host drastically, favoring more resistant bacteria, in particular Gram-positive species, as is seen in our study for *E. incisus* and *E. variegatus*. A third hypothesis is based on results obtained of *D. citri* and '*Candidatus* Liberibacter asiaticus' by Vyas *et al*. (2015): this study demonstrated that the phytopathogen could modulate free amino acids availability by interfering with hexamerin storage pathways by regulating expression of amino acid storage protein genes. Such evidence suggests that the reason why there is a dramatic reduction in genus *Sulcia*, which is heavily committed to amino acid production and encodes enzymes for synthesis of all amino acids required as animal nutrients, is simply due to the fact that an infected insect does not need such a high abundance of this bacterial genus. On the other hand, sap-feeding insects rely heavily on the contribution of their obligate symbionts to maintain their metabolism (McCutcheon and Moran 2007). For this reason, the loss of dominance by the beneficial *Sulcia* endosymbionts could instead prove to be detrimental to the insect's fitness. More data on the fitness of the infected and non-infected insects would be needed to give a correct interpretation of this result. Genus *Wolbachia* tended to be present only in the non-infected specimens and was largely reduced in the infected insect species, except in the case of *D. europaea*, in which the abundance of *Wolbachia* was higher in the '*Ca*. P. solani' infected group. From these results, it becomes evident that the interaction is not just between the phytoplasma and *Wolbachia*, but that the insect species and the rest of the microbiota play a role in determining its outcome. Still, considering that co-presence of phytoplasma and *Wolbachia* was not observed in the majority of the insect species, in general it is reasonable to conclude that a negative interaction

governs the relationship between phytoplasma and *Wolbachia*. It should be established whether phytoplasma infection affects the *Wolbachia* concentration or if the presence of *Wolbachia* confers protection either by reduction in pathogen load, or competition with the pathogen (Krstić *et al*. 2018).

CONCLUSION

This study described the bacterial communities associated with seven insect species hosting '*Ca*. P. solani' and found in vineyards in North Italy. The mutualistic endosymbiont *Sulcia* was found as the prevalent member of the microbiota in all insect individuals non-infected by the phytoplasma. The non-vector *C. viridis* carries unique bacterial signatures (i.e, *Sodalis*, *Cronobacter*) distinguishing its microbiota from that of vector insects, including its fellow xylem-feeder *P. spumarius*.

Beta-diversity analysis revealed that the xylem-feeding behavior of *C. viridis* and *P. spumarius* gave a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. Anyway, the aforementioned unicity of *C. viridis* reinforces the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Analyses highlighted that, in North Italy, phytoplasma infection (not related to its abundance) is associated with major change due to an increase of diversity in the microbiota structure exclusively in *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, the only species, among the analyzed ones, belonging to the subfamily Deltocephalinae.

Considering the specific bacterial genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), obtained data showed that '*Ca*. P. solani' may have an adverse effect on the presence of *Sulcia* as well as *Wolbachia*, while *Dyella* was not found. Further studies are necessary to elucidate whether observed differences (reduction of *Sulcia* and *Wolbachia*, and increase of bacterial diversity) in phytoplasma infected insects are associated with fitness increase or decrease.

The results of this study indicate an interesting perspective regarding the microbial signatures that could be relevant to determine whether an insect species can be a vector or not, opening up new avenues for developing MRM-based approaches to contain BN spreading.

FUNDING

This work was partially funded by Consorzio per la Tutela del Franciacorta.

CONFLICT OF INTEREST

None declared.

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Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

Abdelhameed Moussa[1], Alessandro Passera[1], Francesco Sanna[2], Monica Faccincani[3], Paola Casati[1], Piero Attilio Bianco[1], Nicola Mori[2,4], Fabio Quaglino[1,*,†]

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FIGURE LEGENDS

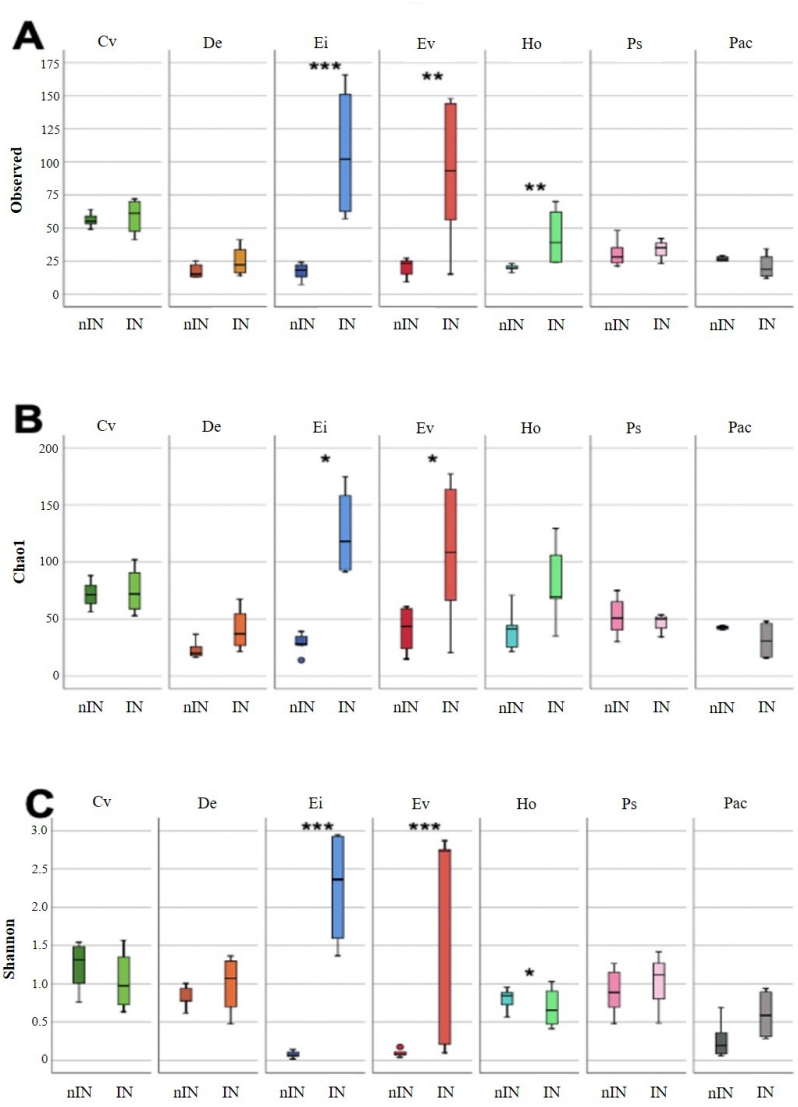
Figure 1. OTU richness in insect microbiomes. Alpha diversity (Observed, Chao1, Shannon) comparison among '*Ca*. P. solani' infected and non-infected insect species. Cv: *Cicadella viridis*; De: *Dictyophara europaea*; Ei: *Euscelis incisus*; Ev: *Euscelidius variegatus*; Ho: *Hyalesthes obsoletus*; Ps: *Phylaenus spumarius*; Pac: *Psammotettix alienus*/*confinis*. nIN: non-infected; IN: infected. Significance level: * < 0.05; ** < 0.01; *** < 0.001.

Figure 2. Venn diagrams showing the comparative distribution of OTUs in the different '*Ca*. P. solani' infected and non-infected individuals within insect species. nIN: non-infected; IN: infected.

Figure 3. Differential heat tree showing differences in bacterial composition to the species level. The comparisons were made among the '*Ca*. P. solani' infected and non-infected groups. A, *E. incisus* where the green color represents the microbial community of the infected group and the brown color represents the non-infected group. B, *E. variegatus* where the green color represents the microbial community of the infected group and the brown color represents the non-infected group. For each taxon, a Wilcoxon rank-sum test was used to test for differences.

Figure 4. Beta-diversity. Graphs reporting the distribution of the samples according to beta-diversity calculated with a Bray-Curtis distance index. Different shape of the markers indicates different '*Ca*. P. solani' infection status, different colors indicate different insect species, as indicated in the legend. Cv: *Cicadella viridis*; De: *Dictyophara europaea*; Ei: *Euscelis incisus*; Ev: *Euscelidius variegatus*; Ho: *Hyalesthes obsoletus*; Ps: *Phylaenus spumarius*; Pac: *Psammotettix alienus*/*confinis*.

Figure 5. Relative abundance of operational taxonomic units at different levels: (A) phylum, (B) genus. Cv: *Cicadella viridis*; De: *Dictyophara europaea*; Ei: *Euscelis incisus*; Ev: *Euscelidius variegatus*; Ho: *Hyalesthes obsoletus*; Ps: *Phylaenus spumarius*; Pac: *Psammotettix alienus*/*confinis*.



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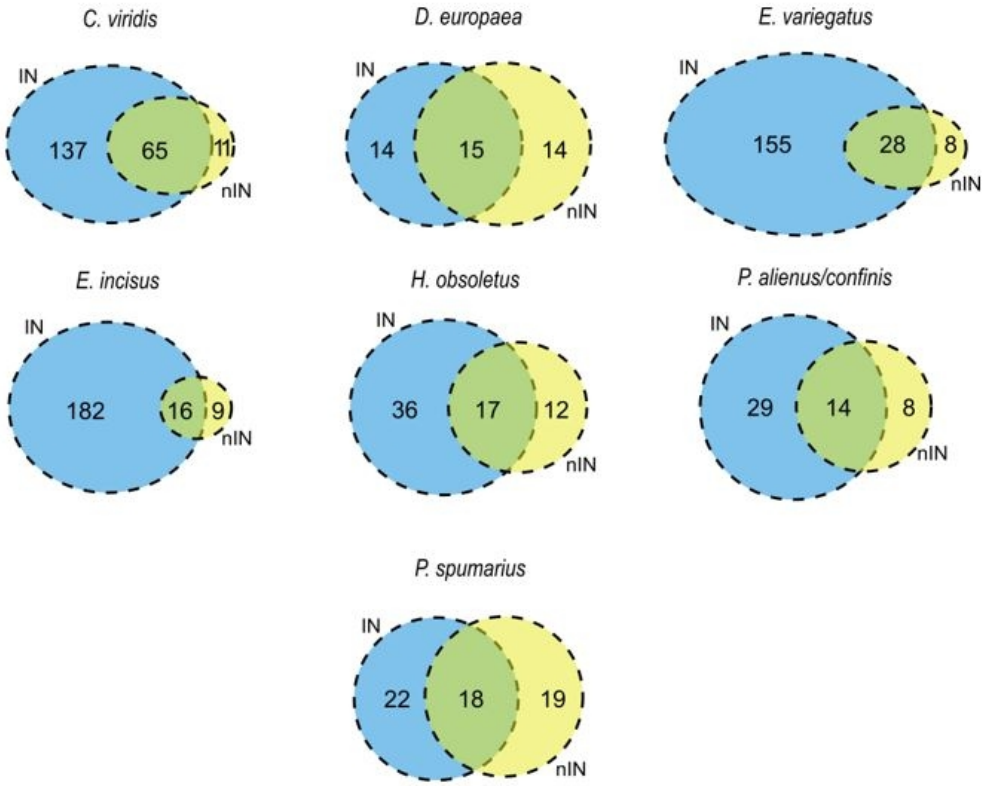
182x260mm (150 x 150 DPI)



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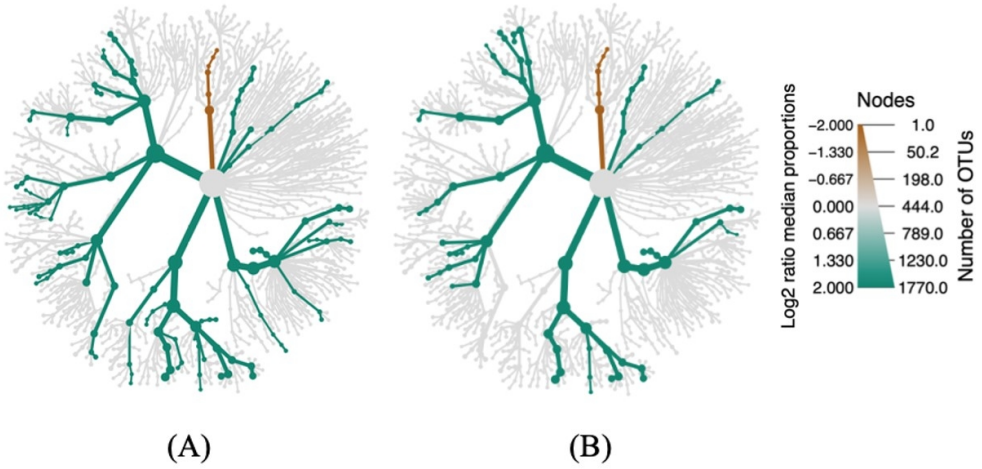
178x143mm (96 x 96 DPI)



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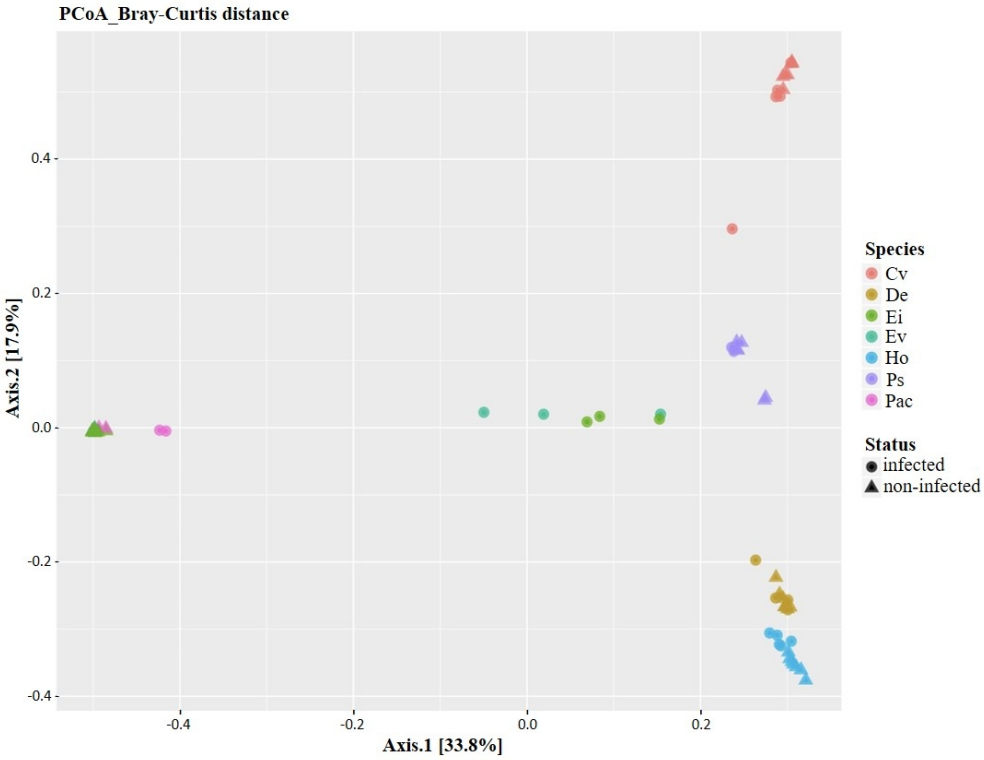
324x159mm (96 x 96 DPI)



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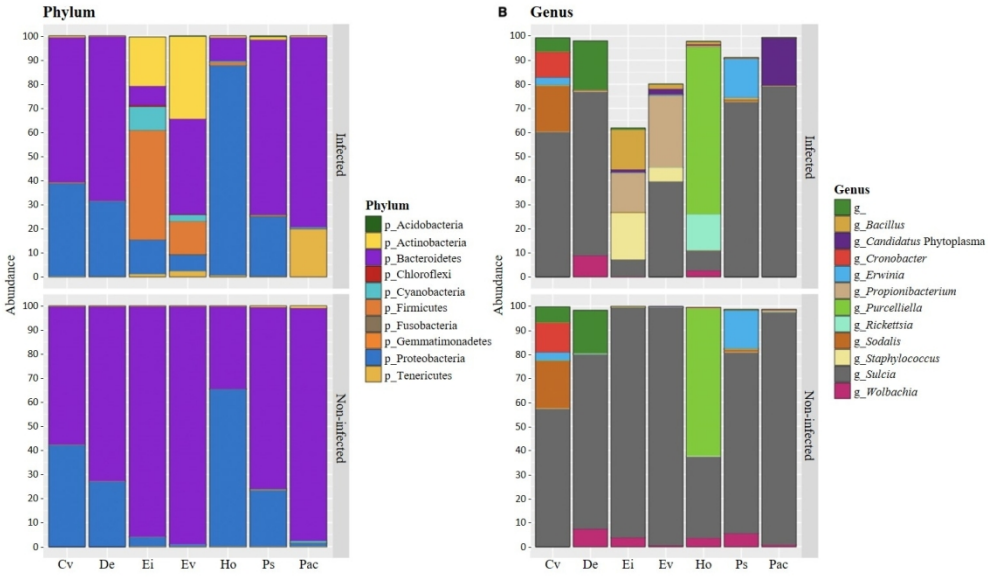

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267x156mm (150 x 150 DPI)

Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

Abdelhameed Moussa[1,2,#], Alessandro Passera[1,#], Francesco Sanna[3], Monica Faccincani[4], Paola Casati[1], Piero Attilio Bianco[1], Nicola Mori[5], Fabio Quaglino[1,*,†]

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TABLES

Table 1. Insect species abundance, infection rate by '*Ca*. P. solani', and selected specimens for NGS analyses.

Insect	No. specimens collected	No. specimens CaPsol-infected	Infection rate (%)	No. specimens used for NGS (healthy/infected)	No. specimens analyzed after NGS (healthy/infected)
Aphrodes makarovi	6	0	0	0	0
Cicadella viridis	39	13	33	8/6	8/5
Dicranotropis hamata	8	0	0	0	0
Dictyophara europaea	40	15	38	9/5	9/4
Euscelidius variegatus	75	32	43	7/7	7/5
Euscelis incisus	59	18	31	9/5	9/4
Hyalesthes obsoletus	47	21	45	7/7	7/5
Laodelphax striatella	16	0	0	0	0
Philaenus spumarius	39	16	41	7/7	7/3
Psammotettix alienus/confinis	71	12	17	6/6	6/4

Table 2. Number of reads and OTUs produced for infected and non-infected group of the different insect species

Species	Status	No. samples	Reads	OTUs
C. viridis	Infected	5	96729	202
	non-infected	8	143399	76
D. europaea	Infected	4	12803	29
	non-infected	9	25528	29
E. incisus	Infected	4	37284	198
	non-infected	9	33499	27
E. variegatus	Infected	5	34157	183
	non-infected	7	28210	36
H. obsoletus	Infected	5	30474	53
	non-infected	7	36010	29
P. spumarius	Infected	3	5609	40
	non-infected	7	12946	37
P. alienus/confinis	Infected	4	11134	43
	non-infected	6	19684	22

TABLE 3. Relative abundance (%) of '*Ca*. Phytoplasma', *Sulcia* and *Wolbachia* OTUs

Insect	Status	'*Ca*. Phytoplasma'	*Sulcia*	*Wolbachia*
C. viridis	Non-infected	0.00	57.49	0.00
	Infected	0.04	59.29	0.00
D. europaea	Non-infected	0.00	76.05	8.24
	Infected	0.08	69.36	9.69
E. incisus	Non-infected	0.00	95.78	3.91
	Infected	1.41	6.88	0.00
E. variegatus	Non-infected	0.00	98.62	0.56
	Infected	3.33	41.42	0.00
H. obsoletus	Non-infected	0.00	32.99	3.26
	Infected	0.87	7.35	2.33
P. spumarius	Non-infected	0.00	74.10	5.65
	Infected	0.08	71.84	0.02
P. alienus/confinis	Non-infected	0.00	96.26	0.87
	Infected	21.48	78.34	0.16

Point-by-point reply

Editor comments

Comment 1. Abstract and Introduction. Please introduce the topic more broadly for a more general audience. Terms like grapevine yellows must be explained and simple facts like phytoplasmas being bacteria must be mentioned to make your work accessible to a broader readership.
Answer 1. As suggested, we expanded the description of grapevine yellows in the abstract (lines 24-27) and introduction (lines 59-65).

Comment 2. Figure 1: the legend is very short. Readers should be able to understand the figure without reading the main text. So please specify the species names, the significance levels and what we are looking at here in general (i.e. OTU diversity in insect microbiomes).
Answer 2. We modified the legend as suggested (File "Figure Legends", lines 18-22).

Comment 3. Figure 2: I have two comments. First would it not make sense to have two trees to contrast infected vs. non-infected individuals? Otherwise this figure is not very informative apart from showing many taxa. Second, some of the labels are extremely small and eligible. This must be fixed.
Answer 3. Considering your comment, we preferred to delete this figure and maintain the
Figure 4, renumbered as Figure 3 in the revised manuscript, including two trees comparing the bacterial microbiota associated with infected vs non-infected individuals of the insects *E. incisus* (A) and *E. variegatus* (B).

Comment 4. Figure 3: Please extend the figure legend to include names of the insect species. Moreover, the axis labels are so small that they cannot be read properly. The same holds true for Figure 5 legend.
Answer 4. We modified the legends and the labels of both Figure 3 (renumbered as Figure 5) (File "Figure Legends", lines 41-44) and Figure 5 (renumbered as Figure 4) (File "Figure Legends", lines 34-39).

Comment 5. Figure 6 looks good, but I'm a bit confused. Here, it seems that the microbiomes are different between infected vs. non-infected species, while the previous analysis in Fig. 3 and 5 show that in most cases there are no significant differences. I understand that the analysis look at slightly different parameters of the microbiome. Nonetheless, the results should correlate, shouldn't they? In any case, the differences between the analysis and their implications must be discussed carefully. Clearly something is going on at the OTU level. Across the insect species, it seems that infected individuals have higher OTU numbers, and a certain fraction of OTUs present in the non-infected individuals disappear.
Answer 5. We agree with you about differences observed at OTU level. It is important to consider that this analysis is only qualitative. The results observed regarding the

number of different OTUs in infected and non-infected insect individuals do not correspond to a change in microbiota structure (beta diversity analysis, renumbered Figure 4) because the majority of OTUs included in Venn diagram are poorly represented within the microbiota. Furthermore, some of these "unique" OTUs in Venn diagram belong to the same phylum/genera, and therefore they are included in the same group of abundance analysis (renumbered Figure 5).
For clarity, in order to eliminate this apparent contradiction, we changed the order of presentation of the results. We switched the order of the paragraphs to present first all the data related to descriptive analysis (alpha diversity, Venn diagrams), and then the quantitative ones (beta diversity, abundance). This flow reasoning was explained in the Results (lines 301-306) as follows: "Venn diagram representation showed a qualitative difference among OTUs identified in infected and non-infected individuals in the species, without considering the vital quantitative aspect in describing the community structure. To compare the microbial community structure among the '*Ca*. P. solani' infected and non-infected individuals within and among insect species, principle coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 4)".

Comment 6. Figure 6 legend. Again, there is not enough information provided. The labels IN and nIN must be explained. Moreover, this figure does not show infected versus non-infected species as is currently stated, but infected vs. non-infected individuals within a species.
Answer 6. We modified the legend of Figure 6, renumbered as Figure 2, as suggested (File "Figure Legends", lines 24-25).

Comment 7. Line 225: Please show the data.
Answer 7. We deleted "data not shown" because it was wrong. The results were already included in the text as follows: "Bacterial 16S rDNA fragment 27F/1492R was amplified by the TNAs extracted from all insect specimens and not in the negative control, evidencing the TNAs suitability for further molecular analyses" (lines 243-245).

Comment 8. Lines 366+367: It is unclear what these several considerations are. The text simply goes on afterwards. If you want to highlight key considerations then it would probably make sense to number them. e.g. First, alpha diversity… Second, …
Answer 8. Reading the manuscript carefully, we noticed that this sentence was unnecessary and inappropriate. Thus, we deleted it in the revised text.

Comment 9 (minor comments).
- Line 25: Remove "therefore" because there is no logic link to the preceding sentence.
- Line 45: Change to "depend for food".
- Line 221: This reads odd, as you cannot study species, which have not been captured. Please revise.
Answer 9. We fixed the text in accordance with these comments.

Reviewer 1 Comments

Comment 10. An important point regards the insect species selection. Most of them were previously reported vectors of phytoplasmas, but they show different feeding behaviors. Specifically, P. spumarius is a well-known xylem feeder, whereas the other vector species are phloem feeders; moreover, the selected non-vector species, namely C. viridis, is a xylem feeder as well. Both P.s. and C.v. were proposed to occasionally ingest phloem sap, getting in touch with the phytoplasma as demonstrated by the detected field infection. Nevertheless, their different feeding behavior could have influenced the overall microbiota composition, and this should be discussed. Moreover, since Cv is used a non-vector control, the occurrence of possible differences in the bacterial community related to the divergent vector status should be commented, otherwise there is no point in including this species in the study.

Answer 10. We would like to thank the reviewer for this comment. We completely agree with the necessity to (i) explain the selection of insect species analyzed in the study, with particular attention for *C. viridis*; (ii) compare between xylem- and phloem-feeders and discuss appropriately the observed differences; (iii) compare between vectors and non-vectors considering also their feeding behavior. To insert these considerations within the text, we modified part of introduction (lines 135-142), results (lines 361-366), discussion (467-472), and conclusions (519-525).

Comment 11. Finally, the phytoplasma infection rates found in the xylem feeders (including the non-vector Cv) are fairly high, if we consider that they are supposed to ingest phloem sap only occasionally. Please discuss such findings.

Answer 11. We agree with this comment: the phytoplasma infection rate is unusually high, particularly for xylem-feeders but also for phloem-feeders. Anyway, we found the same trend during the survey carried out on the same insects in the same area in years from 2013 to 2016. Thus, in the Discussion, we stated that "The insect survey and molecular identification of '*Ca*. P. solani', conducted in this study, confirmed the presence of abundant populations and the unusually high infection rate (>10%) in 2018 for the main vector *H. obsoletus* and for a majority of the insect species recently reported as vectors (Quaglino *et al*. 2019)" (lines 369-372).

Comment 12. Another point regards the interpretation of the role of Wolbachia in the considered insect species. All throughout the text, Wolbachia is mentioned as an exclusively harmful bacterium for insects; however, there is plenty of publications of mutualistic roles for this bacterium in many insects. Even its role as a biocontrol agent is not mainly related to an entomopathogenic activity, but rather to antagonistic activity against pathogens or release of incompatibility inducing strains in Wolbachia-free populations. Moreover, the actual role of Wolbachia in the mentioned species is still unclarified, and it is well-known that the interaction between Wolbachia strain and host genotype may strongly influence the final phenotypic effect in the host. So, the sentences regarding the role of Wolbachia should be mitigated in the text, taking into account that the bacterium could still be beneficial at least for some of these insects. Also, when

treating the interaction between Wolbachia and phytoplasma in the host microbiota, the authors take for granted that the observed mutual exclusion derives from phytoplasma inhibition of Wolbachia, but it could also be the opposite. For instance, in many mosquitoes, Wolbachia has an antagonistic effect against the vectored pathogen. Also, it is interesting to note that the only species where the mutual exclusion is not detected is D. europaea, where previous work demonstrated a mutual exclusion with another phytoplasma (Krstic et al 2018, https://doi.org/10.1111/aab.12400).
This work should be cited in the corresponding section.

Answer 12. We thank the reviewer for these insights and suggestions on *Wolbachia*. Considering these points, we expanded the sections regarding *Wolbachia* and its role, in particular the abstract (lines 33-35), introduction (lines 107-119), the results (lines 356-360), discussion (line 388; lines 503-513), and conclusions (lines 530-534). Concerning the study by Krstic and colleagues, suggested by the reviewer, we considered their results and hypotheses in the role of *Wolbachia* in *D. europaea* as vector of flavescence dorée phytoplasma, and cited this work accordingly. However, we did not directly compare our results with those by Krstic because our study focused on a quantitative bacterial microbiota analysis conducted on insects sampled in a single location, while those reported by Krstic are qualitative analyses carried out on insects sampled in various locations in different countries, with a main focus on the epidemiological significance of the presence of *Wolbachia* in different populations. These differences in methodology, aim, and scope made it impossible, in our opinion, to compare the results of the two studies. Also, our results are not in disagreement with those by Krstic as also in that study (supplementary tables) some individuals infected both by *Wolbachia* and phytoplasma are reported.

Comment 13. An additional critical aspect regards the conclusion drawn on the observed effect on microbiota composition related to phytoplasma infection. The 3 species where a significant change was noted are all in the Cicadellidae subfamily deltocephalinae. This group is well-known to host symbiotic Nasuia as a co-primary symbiont together with Sulcia, conversely Nasuia was not found in this study. The absence of Nasuia may be due to TNA- or PCR-related biases, and this could have influenced the final result, please discuss.

Answer 13. We thank the reviewer for this comment regarding the Deltocephalinae family and their endosymbiont *Nasuia*. We agree that all steps of the analysis, from extraction of nucleic acids to the sequencing with Illumina may present biases that can affect the final results presented. However, considering that *Nasuia* was not found in any sample from Deltocephalinae, we can conclude that any possible methodological bias affected all the samples equally, and therefore does not invalidate the conclusions that the microbiota is restructured in the infected individuals of this subfamily. In fact, while we cannot exclude a bias being present in our analyses, it has been previously reported that not all members of the Deltocephalinae subfamily host *Nasuia* in their microbiota, and that the presence, abundance, and quality of the mutualistic endosymbionts can be influenced by environmental conditions, and even ancient endosymbionts such as *Nasuia* can be replaced (Nishino et al. 2016; Brentassi et al. 2017; Kobiałka et al. 2018; Mao and Bennett 2020). In one such cases, like in our data, the Deltocephalinae insects that

were missing *Nasuia* showed an increased abundance of *Sulcia* (>95%) instead (Brentassi et al. 2017). For these reasons, we consider that our results are in line with those previously reported in literature.
Following the indications presented in this comment, we inserted new paragraphs in the Discussion (and related references), including these considerations regarding the role and absence of *Nasuia* (lines 441-459).

Comment 14. Finally, the conclusion drawn on the possible reduction of Sulcia density in response to phytoplasma infection should be mitigated as well. The authors suggest that "reduction in the abundance levels of 'Ca. Sulcia muelleri' in the 'Ca. P.solani' infected insect vectors may be a marker of the increased fitness of the insect, indicating that the host becomes more efficient in utilizing its metabolic resources" (L466-469). However, Sulcia is highly conserved in the Auchenorrhyncha because it provides the hosts with essential amino acids that are missing in their diet and are not self-produced by the insect itself. So, to justify the statement, the phytoplasma should directly provide the insect with these amino acids, or at least favoring the establishment of other mutualists capable of doing so. The authors should either provide evidence for this assumption or remove the sentence, and limit speculations on this aspect everywhere in text.
Answer 14. We agree with the reviewer, the conclusion on the role of the reduction of *Sulcia* was indeed too speculative. We mitigated the sentences regarding this phenomenon throughout the text (lines 33-35; lines 495-502), and stated that further data regarding the fitness of the insects should be obtained before a definitive interpretation of this result can be given (lines 530-534).

Comment 15 (minor comments).
- L54: replace "Auchenorrhycha" with "Auchenorrhyncha and Sternorrhyncha".
- L98: remove "including histidine and methionine" (these amino acids are usually supplied by co-primary symbionts and not by Sulcia).
- L179-180: add the information on the hypervariable region amplified (V4).
- replace "v4-v5" with "v4".

Answer 15. We fixed the text in accordance with these comments.

Reviewer 2 comments

Comment 16. Line 30- needs rephrasing. It is not clear: 1. if the host utilizes better its metabolite in the presence of phytoplasma or not. This is a hypothesis that was not the objective of the study nor was it proved in the study. 2. regarding Wolbachia- this is also a hypothesis and not a conclusion from this study. This point must be addressed in discussion as well.
Answer 16. In accordance to other changes carried out throughout the text to correct the concerns about *Wolbachia* raised by the reviewers and editor [introduction (lines 107-119), the results (lines 356-360), discussion (line 388; lines 503-513), and conclusions (lines 530-534)], the abstract of this manuscript was extensively modified. The improved

version of the abstract includes a mitigation of the sentences regarding the role of *Sulcia* and *Wolbachia* (lines 33-35).

Comment 17. Line 116 - add full name of concept NGS not just abbreviation
Answer 17. We included the full-length name of the concept, as indicated (lines 127-128).

Comment 18. Line 135- explain why you did not include R. panzer in the study.
Answer 18. In the introduction, a more detailed explanation on the selected species was added. In particular, we added a sentence regarding *R. panzeri* (lines 141-142), specifying that it was not considered for the analysis as it is not an insect found in the vineyards of the examined area.

Comment 19. Line 272 – regarding fig 3A: the authors need to explain why the phylum Tenericutes is not found in all phytoplasma infected species especially Ho
Line 286-refer to fig 3B – explain why no phytoplsama OTU were found in phytoplasma harbored in all specimens especially the species with high phytoplasma rates according to PCR, e.g. Ho Ps, and Cv. Also refer to the point that there is no correlation between PCR and OTU results of phytoplasma.
Answer 19. Figures 3A and 3B show the OTUs as relative abundance and, for this reason, could be misleading regarding the presence of phytoplasma OTUs in the microbiota. In fact, OTUs assigned to phytoplasma are present in all the infected samples, albeit in most cases with less than 1% abundance. To clarify this point, we added a new table in the text (Table 3), showing the relative abundance of bacterial genera relevant for our study ('*Ca.* Phytoplasma', *Sulcia*, *Wolbachia*), showing that there is correlation with PCR results, as the samples determined to be infected by PCR are also those harboring phytoplasma OTUs (lines 354-366).

Comment 20. Line 293 - the effect of phytoplasma on whole microbial community is presented for Ev and Ei. Fig 3 shows a smaller change for Ho. Why results are not shown also Ho, being the main vector of BN?
Answer 20. The results regarding the change in bacterial community for *H. obsoletus* are reported a few lines before those for *E. variegatus* and *E. incisus* (lines 346-348).

Comment 21. Line 331- "No single OTU is shared among all species, nor among all infected or non-infected samples, indicating that the bacterial communities in these populations are clearly distinct and do not share a common core." This sentence is not clear. Explain on what basis this statement is stated.
Answer 21. The statement refers to the comparison of OTUs present in samples, the same dataset that is used to produce the Venn diagrams (renumbered Figure 2). To clarify the sentence, we rephrased it as follows "The bacterial communities in these insect populations are clearly distinct and do not share a common core as no single OTU is shared (i) among individuals of all insect species regardless of infection, (ii) among infected individuals regardless of insect species, (iii) among non-infected individuals regardless of insect species" (lines 281-284).

Comment 22. Line 334- on what figure the description is based on? For example- from fig 3B, Propionibacterium is not shared by Ev infected and not infected, same is for Rickettsia in HO. please correct the description.
Answer 22. The statement is based on the OTUs included in the Venn diagram (renumbered Figure 2), and it is a description of the OTUs that make up the number of shared OTUs between infected and non-infected individuals in the same species. To avoid confusion between this descriptive analysis and the quantitative analysis in the interpretation of the text, the order of the results has been changed, presenting the Venn diagrams and the description of shared OTUs before the quantitative results.

Comment 23. Fig 1. Line 248- "The Shannon index, indicating the evenness of species distribution ranges from a minimum of 0.089 in non-infected E. variegatus, to a maximum of 2.25 in infected E. incisus." Explain how these indices were calculated regarding the scale in fig 1C.
Add the statistical meaning of * or ***.
Also- 65 and 82 are posted in the fig. near EI and EV non-infected Shanon and Chao-1 columns. What is the meaning of these numbers?
Answer 23. We fixed the scale in y-axis of Figure 1C. We deleted the numbers 65 and 82 from the Figure (they were inserted for a mistake). The legend of Figure 1 was modified indicating the species acronyms and the statistical meaning of *, **, *** (File "Figure Legends", lines 18-22).

Comment 24. Fig 2 –the font of species names is too small to read.
Answer 24. In accordance with the comments raised by the Editor, we preferred to delete this figure and maintain the Figure 4, renumbered as Figure 3 in the revised manuscript, including two trees comparing the bacterial microbiota associated with infected vs non-infected individuals of the insects *E. incisus* (A) and *E. variegatus* (B).

Comment 25. Line 432 -from fig 3B, there is dramatic decrease in Sulcia in Ho, Ev, Ei. In the other 3 species (Cv, De, Ps), there is no big difference in abundance of Sulcia between infected and non infected. Also the change in Pac is rather small. However the number of phytoplasma OTU's in Pac were the highest. Please refer to this point.
Also, phytoplasma OTU were not observed in Ho, the authors have to explain their conclusion that the reduction abundance of in Sulcia and Wolbachia is related to phytoplasma presence.
Answer 25. To clarify these points, we added a new table in the text (Table 3), showing the relative abundance of bacterial genera relevant for our study ('*Ca.* Phytoplasma', *Sulcia*, *Wolbachia*), showing that there is correlation between phytoplasma presence and decrease of *Sulcia* and *Wolbachia* (Results, lines 354-366; Discussion, line 439).

Comment 26. The main concern is that phytoplasma was shown by PCR in 7/10 species but only in 3/10 by OTUs. Also phytoplasma was not detected by OTU in Ho which is the main vector of BN and showed the highest rate of infection by PCR. Please refer to this point.

Answer 26. Please, see answer to Comment 19.

Comment 27. The author need to explain why it is concluded that a reduction in Sulcia in infected insects is a marker for increased efficiency in metabolite utilization of the insect. From the same evidence it can be explained that the fitness is reduced in the presence of phytoplasma because the abundance of Sulcia in reduced.
Answer 27. We agree with the reviewers, the conclusion on the role of the reduction of *Sulcia* was indeed too speculative. We mitigated the sentences regarding this phenomenon throughout the text (lines 33-35; lines 495-502), and stated that further data regarding the fitness of the insects should be obtained before a definitive interpretation of this result can be given (lines 530-534).

Comment 28. line 471- this is an hypothesis that has to be proved, not a conclusion
Answer 28. Considering the points raised by the reviewers, we expanded the sections regarding *Wolbachia* and its role, including the conclusions (lines 530-534).

Bacterial ~~M~~microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

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One sentence summary: This study describes the microbial community associated with insect vectors of Bois noir disease of grapevine in relation to presence/absence of its etiological agent, '*Candidatus* Phytoplasma solani'.

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ABSTRACT

Bois noir is ~~the prevalent~~a ~~-~~grapevine disease causing severe yield loss in vineyards worldwide. ~~in the grapevine yellows complex.~~ It is associated with '*Candidatus* Phytoplasma solani', a phloem-limited bacterium transmitted by polyphagous insects. Due to its complex epidemiology, it is difficult to organize effective containment measures. ~~Therefore, t~~This study aimed to describe the bacterial microbiota associated with '*Candidatus* Phytoplasma solani' infected and non-infected insect hosts and vectors to investigate if phytoplasma presence can shape the microbiota, with special attention for bacteria known as essential for insect survival, parasites, or phytoplasma antagonists. Alpha-diversity analysis showed a low microbiota diversity in these insects, in which few genera were highly abundant. Beta-diversity analysis revealed that the xylem- and phloem-feeding behavior influences the microbiota structure. ~~highlighted that the phytoplasma presence did not cause a microbiota restructuring in most of these insects.~~Moreover, it highlighted that phytoplasma infection is associated with a restructuring of microbiota exclusively in

Deltocephalinae insect vectors.- Obtained data showed that '*Candidatus* Phytoplasma solani' may have adverse effects on the ~~obligate~~ endosymbionts *Sulcia* and ~~, indicating that the host becomes more efficient in utilizing its metabolic resources, as well as the facultative endosymbiont~~ *Wolbachia*, suggesting a possible fitness modification in the insects~~, suggesting that the phytoplasma can protect the insect from its possible detrimental effects~~. The phytoplasma-antagonistic *Dyella* was not found in any of the examined insect species. The results indicate an interesting perspective regarding the microbial signatures associated with xylem- and phloem-feeding insects, and determinants that could be relevant to establish whether an insect species can be a vector or not, opening up new avenues for developing microbial resource management-based approaches.

Keywords: ~~*Vitis vinifera*;~~ grapevine yellows; *Wolbachia*; *Sulcia*; microbial resource management; phloem-limited bacteria

INTRODUCTION

Diseases that are transmitted by vectors are not only a threat to human health, but can also cause disastrous losses in agriculture, being a threat for livestock and plants upon which we depend ~~to produce~~for food. Most of the vectors that transmit diseases are arthropods, among which insects and mites can transmit a wide range of pathogens to a broad range of hosts (Ciancio 2016).

Among the plant pathogens that are transmitted by vectors, phytoplasmas deserve a specific mention due to their unique nature, being obligate bacterial pathogens with a broad host range that localize in the phloem of their host plant. However, they have a much stricter specificity when it comes to their insect vectors, as several molecular recognition stages are needed for the phytoplasmas to pass from the insect gut to the hemolymph and ultimately to the salivary glands of the vector, from where they can infect new plants (Namba 2019).

Each phytoplasma can have different vectors but all known vectors are insects belonging to the order Hemiptera, suborder Auchenorrhyncha and Sternorrhyncha~~Auchenorrhynca~~, in particular leafhoppers (family Cicadellidae), planthoppers (superfamily Fulgoroidea), and psyllids (superfamily Psylloidea) (Weintraub and Beanland 2006; Alma *et al*. 2015). This study focuses on '*Candidatus* Phytoplasma solani', associated, among others, with grapevine Bois noir, the most widespread disease in the complex of grapevine yellows ~~disease~~(Quaglino *et al*., 2013). This complex includes grapevine diseases, associated with genetically and biologically distinct phytoplasma species, that induce common symptoms (desiccation of inflorescences, berry shrivel,

leaf discolorations, reduction of growth, and irregular ripening of wood), and cause ~~, the most prevalent disease in the complex of grapevine yellows that causes~~ serious economic damage and yield loss in vineyards (Belli *et al.* 2010; Angelini *et al*. 2018~~; Quaglino *et al*. 2013~~).

The epidemiological cycle associated to Bois noir is extremely complex and was recently discovered to include not only the most well-known vectors *Hyalesthes obsoletus* (Maixner 1994) and *Reptalus panzeri* (Cvrkovic *et al*. 2014), but also other eight species: *Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatella*, *Phylaenus spumarius*, and *Psammotettix alienus*/*confinis* (Quaglino *et al*. 2019).

Since the cycle includes so many highly polyphagous insects~~, all highly polyphagous,~~ and a very broad range of secondary~~,~~ wild hosts, it is difficult to organize effective prevention and containment measures (Bertaccini *et al.* 2014; Moussa *et al*. 2019; Quaglino *et al.* 2019). Moreover, ~~as~~ the typical management strategies for phytoplasma diseases, based on the control of the vector with insecticides and the removal of infected plants (Bianco *et al*. 2011), are not effective against '*Ca.* P. solani' (Angelini *et al*. 2018). For this reason, other methods are being envisioned, including the use of Microbial Resource Management (MRM).

MRM is the proper management of the microbial resources available in a given ecosystem in order to solve a practical problem by directing the potential of microorganisms. In particular, ~~and,~~ on the topic of control of insect vectors, some first steps have already been taken towards defining the composition and functionality of microbial communities associated with insects (Marzorati *et al.* 2006; Miller *et al.* 2006; Crotti *et al.* 2012).

Insects, like all other ~~higher organisms~~animals, maintain several symbiotic interactions with their associated microbial community, which has a great influence on their fitness, evolution, and diversity (Margulis and Fester 1991; Ruby *et al*. 2004). The microbial community can contain beneficial symbionts, called mutualists, but also detrimental ones, which are parasites or pathogens, and the dynamic balance found in a microbial community can produce either a positive or negative effect for the health of the host (Berg *et al.* 2014; Lebeis 2014). An MRM approach to control ~~these~~ insect vectors would therefore be performed by manipulating their microbial community ~~of these insects~~ to promote the effect of naturally present antagonistic microorganisms (Trivedi *et al.* 2016).

A negative prospect for this strategy is that, as the interactions between environment, host, and microbiota are very complex and influenced by several variables (Trivedi *et al.* 2015~~)~~; Douglas 2015; Fonseca-García *et al.* 2016), more studies need to be conducted in the description of the bacterial community associated to these vectors before its manipulation can become a viable option. The positive prospect ~~is that~~, since these phloem-feeding insects rely heavily on obligate bacterial

symbionts to provide nutrients which are lacking in their unbalanced diet (Buchner 1965; Baumann 2005; Bourtzis and Miller 2006; Skidmore and Hansen 2017), ~~it~~ is that ~~hypothesized that~~ these insects will be particularly susceptible to unbalances in their microbial community.

A main actor in these obligate mutualistic interactions is '*Candidatus* Sulcia muelleri', a bacterial species that greatly reduced its genome as it evolved as an obligate symbiont~~,~~; moreover, it ~~and~~ is documented to be strictly associated to leafhoppers and planthoppers, among other hosts (Moran *et al.* 2005; McCutcheon *et al.* 2009). This bacterial species is involved in the synthesis of several amino acids necessary for the insect host~~, including histidine and methionine~~ (McCutcheon and Moran 2007). Other mutualistic bacteria involved in these interactions belong to the genera *Nasuia* and *Sodalis* (Kobiałka *et al*. 2018).

Another bacterial genus interesting for MRM approach is ~~On the other side of the spectrum, we can find parasitic or antagonistic bacteria, such as those belonging to the genera *Wolbachia* and *Dyella*.~~ *Wolbachia*, ~~is a genus of~~ ubiquitous ~~bacterial~~ endosymbionts associated with over 60% of known insect species, as well as other arthropods and nematodes (Hosokawa *et al.* 2010; Zug and Hammerstein 2012; Newton and Rice 2020). *Wolbachia* species are cytoplasmically inherited and known as reproductive parasites due to their ability to manipulate reproduction such as sperm-egg incompatibility (cytoplasmic incompatibility), parthenogenesis induction, male killing, and feminization, making it a possible biocontrol agent against the vectors (Werren 1997; Stouthamer *et al.* 1999; Werren *et al.* 2008; Brelsfoard and Dobson 2009; Chuche *et al*. 2017). Nevertheless, several studies showed that *Wolbachia* can act as mutualistic towards insect hosts, modulating nutrition and immune responses (Hosokawa *et al.* 2010; Iturbe-Ormaetxe *et al*. 2011; Newton and Rice 2020). Moreover, recent studies proposed that *Wolbachia* can act as biocontrol agent of insect-transmitted pathogens, including phytoplasmas, by increasing latency period and blocking pathogen transmission ~~is an interesting and promising biological control agent that can be used to stop or prevent the transmission of several pathogens~~ (Shaw *et al.* 2016; Chuche *et al*. 2017).

Dyella-like bacterium (DLB), gram-negative, aerobic, rod-shaped endophytic bacteria belonging to the family Rhodanobacteraceae, can be acquired by feeding and has shown a potential biocontrol activity against phytoplasmas and their cultivable relative *Spiroplasma melliferum* (Iasur-Kruh *et al.* 2017, 2018). The possible mechanisms of DLB antagonism towards phytoplasmas have been hypothesized to be (i) competition for nutrients or colonization niches, (ii) induction of plant systemic resistance, (iii) secretion of plant growth hormones, or (iv) secretion of phytoplasma growth inhibitory substances (Eljounaidi *et al.* 2016).

In this scenario, the current study aims to characterize through an Next Generation Sequencing (NGS) approach the ~~microbial~~ bacterial community associated with selected ~~recently~~

~~identified~~ '*Ca*. P. solani' insect hosts, ~~vectors (*A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, *P. spumarius*, and *P. alienus*/*confinis*), the main vector *H. obsoletus*, and one insect host but non-vector (*Cicadella viridis*),~~ both infected and non-infected by the phytoplasma, with the following goals: (i) describe the ~~microbial~~ bacterial communities in different insect hosts of '*Ca*. P. solani'; (ii) determine whether the presence of '*Ca*. P. solani' affects the ~~microbial~~ bacterial community, in particular if it can cause a dysbiosis (also called dysbacteriosis) or increase diversity; (iii) evaluate the presence of possible antagonists towards the insect (e.g. *Wolbachia* spp.) or phytoplasma (e.g. *Wolbachia* spp. and *Dyella*-like bacteria); (iv) investigate the effect of '*Ca*. P. solani' on the obligate endosymbiont '*Ca.* Sulcia' spp.. The selected insects are the main vector *H. obsoletus*, newly reported vectors (phloem-feeders: *A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, and *P. alienus*/*confinis*; xylem-feeder: *P. spumarius*), and *Cicadella viridis*, one of the most abundant insects living in Italian vineyard, harboring with high infection rate but not vectoring '*Ca*. P. solani' (Quaglino *et al*. 2019), and characterized by xylem-feeding activity. *C. viridis* was included in the study for comparing the microbiota associated with xylem- and phloem-feeders, and investigating the phytoplasma influence on the microbiota structure in comparison with vectors. *R. panzeri* was not among the selected vectors because it is not found in the studied area.

Achieving the previously mentioned aims regarding the description of the bacterial communities may help in devising MRM-based approaches to achieve the main objective of biological control of '*Ca*. P. solani' and its insect vectors.

MATERIALS AND METHODS

Insect collection

Specimens of the insect species *A. makarovi*, *C. viridis*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius*, and *P. alienus*/*confinis* were captured by sweep entomological net in mid-July 2018 in the Chardonnay organic vineyard (Franciacorta, Lombardy Region, North Italy; N 45°35′38.12″, E 10°09′34.32″) where new insect vectors of '*Ca*. P. solani' had previously been identified (Quaglino *et al*. 2019). Insect individuals were stored in ethanol 90%, transferred to the lab for species identity confirmation by stereomicroscope based on the taxonomic keys of den Bieman *et al.* (2011), and maintained in absolute ethanol at 4°C till use. Regarding the genus *Psammotettix*, given that the dichotomous keys are related only to males, the species *P. alienus* and *P. confinis* were considered together.

Total nucleic acids extraction and suitability for amplification

Total nucleic acids (TNAs) were extracted from ethanol preserved insects (dried by filter paper) through homogenization in a CTAB-based buffer [2% w/v cetyltrimethylammonium-bromide (CTAB); 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HCl pH 8.0; 0.5% ascorbic acid]. After incubation at 60°C for 20 min, TNAs were separated with one volume of chloroform: isoamyl alcohol 24:1 v/v solution and precipitated with the addition of one volume of cold isopropanol. The TNAs pellet was then washed with ethanol 70%, air dried, dissolved in 30µL of TE buffer pH 8.0, and maintained at −20 °C until use (Moussa *et al.* 2019).

The suitability of the extracted TNAs for amplification was tested through a bacterial *16S rRNA* gene PCR assay using the universal primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') / 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). PCR reactions were conducted in Applied Biosystems 2720 thermocycler (Applied Biosystems, Monza, Milan) with the following conditions: 2 min at 95 °C; 35 cycles consisting of 1 min at 95 °C, 1 min 30 s at 50 °C and 2 min at 72 °C; 10 min at 72 °C. PCR reactions were performed in 25 µL volume containing 50 µM of each dNTP, 0.4 µM of each primer, 1.5 mM MgCl2, 1× polymerase buffer, 1 unit GoTaq polymerase enzyme (Promega, Milan, Italy). PCR mixture devoid of TNAs was employed as negative control. PCR products were analyzed by electrophoreses in 1% agarose gel stained with Midori green under a UV transilluminator. Only the samples that gave positive amplification with this reaction were considered for further analyses.

Molecular detection of '*Candidatus* Phytoplasma solani'

The presence of '*Ca*. P. solani' in collected insects was verified by species-specific nested PCR-based amplification of the *stamp* gene using the primer pair *Stamp*-F (5'-GTAGGTTTTGGATGTTTTAAG-3') / *Stamp*-R0 (5'-AAATAAAAGAACAAGTATAGACGA-3'), followed by the primer pair *Stamp*-F1 (5'-TTCTTTAAACACACCAAGAC-3') / *Stamp*-R1 (5'-AAGCCAGAATTTAATCTAGC-3') (Fabre *et al*. 2011). PCR reactions were conducted in Applied Biosystems 2720 thermocycler with the following conditions: 4 min at 94 °C; 35 cycles consisting of 30 s at 94 °C, 30 s at 56 °C (direct PCR) or 52 °C (nested PCR) and 1 min 30 s at 72 °C; 7 min at 72 °C. PCR mixture devoid of TNAs was employed as negative control. PCR reaction mixtures and PCR products visualization were as described above for bacterial *16S rRNA* gene.

Illumina Mi Seq sequencing

Based on the molecular detection of '*Ca*. P. solani' and the requested TNAs quantity (at least 0.5 µg) / quality (ratio 260/280 nm ~2), TNAs extracted from 96 insect specimens were selected to

undergo Illumina Mi Seq sequencing. These 96 samples were picked to ensure that at least five samples for each insect species were included in both the '*Ca*. P. solani'-infected and non-infected groups.

Next generation sequencing library preparations and Illumina Mi Seq sequencing were conducted by an external provider (Personal Genomics, Verona, Italy). The bacterial *16S rRNA* gene hypervariable region V4 libraries ~~was~~ were prepared using the forward primer 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3'), and the amplification of sequences belonging to mitochondria was blocked using a PNA blocker (Lundberg *et al*. 2013). Metagenomic sequencing was performed using the Illumina Miseq 300PE sequencing technology. Obtained reads were deposited in the EMBL-ENA under the project number PRJEB38750.

Processing of high-throughput sequencing data

The raw sequencing reads were initially filtered, to remove low quality sequences, trim primers and Illumina adapters. The initial quality control of the reads was performed with FastQC v0.11.5. Primers were trimmed with the cutadapt tool version 1.14 (Martin 2011) while adapters were trimmed with Sickle version 1.33 (https://github.com/najoshi/sickle) and Scythe version 0.991 (https://github.com/vsbuffalo/scythe). The obtained reads were analyzed using the QIIME 2 pipeline (Bolyen *et al.* 2019) in order to assign them to OTUs. Allocation to OTUs and clustering were performed using uclust with a minimum similarity of 97% (default). Identified OTUs from representative sequences were aligned to Green-genes (http://greengenes.lbl.gov/) using R-studio. Chloroplast and mitochondria (these constituted only 1-2% in some samples) were filtered as well as rare OTUs (i.e., singletons and OTUs < 10). The resulting OTU table was then used for the subsequent analyses.

Diversity and statistical analysis

After quality filtering and rarifying to 1600 sequences per sample, Alpha-diversity indices (Shannon index, ChaoI and observed OTU) were calculated to ensure that enough sequencing coverage had been achieved by using BiocManager package implemented in the R software (R Project 3.0.2; http://cran.rproject.org/). Observed, Chao1 (Chao 1984) and Shannon H' index (Shannon 1948) were considered for the aforementioned features. Alpha diversity indices were compared between different insect species groups ('*Ca*. P. solani' infected or non-infected). Shapiro test was performed for data normality followed by ANOVA in the case of Observed richness whereas Kruskal test was used for Chao1 and Shanon H' index. Welch t-test was carried out to

compare between the infected and non-infected groups of individual species. Beta diversity was assessed by Bray-Curtis (Bray and Curtis 1957). distance matrices and visualized by principal coordinate analysis (PCoA). The PERMANOVA statistical analysis was performed to determine the significance of microbial community differences among the different insect species and infection status with controlled 10^5 permutations. Taxonomic abundance data was calculated using the percentage abundance of OTUs present in the core microbiota. Heat tree was used to plot all the OTUs present in the dataset using the 'metacoder' package. Taxonomic data were plotted using heat trees in which the size and color of tree parts correspond to reads for each taxon as the size of each taxon. It also plots the number of OTUs assigned to each taxon in the overall dataset as color.

RESULTS

Insects collected and '*Ca*. P. solani' infection rate. A total of 400 insect individuals were captured, belonging to all the insect species under study. The most abundant species were *E. variegatus* (75 individuals), *P. alienus*/*confinis* (71) and *E. incisus* (59), while *L. striatella* (16), *D. hamata* (8) and *A. makarovi* (6) were scarcely present (Table 1). Bacterial 16S rDNA fragment 27F/1492R was amplified by the TNAs extracted from all insect specimens and not in the negative control, evidencing the TNAs suitability for further molecular analyses (data not shown). PCR-based amplification of *stamp* gene identified the presence of '*Ca*. P. solani' in 127 out of 400 individuals. Infection rate was >40% in *H. obsoletus*, *E. variegatus*, and *P. spumarius*, >30% in *D. europaea*, *C. viridis* and *E. incisus*, and >10% in *P. alienus*/*confinis*. The phytoplasma was not identified in the least abundant species *L. striatella*, *D. hamata* and *A. makarovi* (Table 1); these latter three species were thus not included in microbiota analyses. For each of the other seven insect species, the number of '*Ca*. P. solani'-infected and -non-infected specimens selected for microbiota analyses is reported in Table 1.

Bacterial diversity analysis

Poor quality sequences were obtained in twelve out of 96 insect specimens that were excluded from further analyses (Table 1). Sequencing of the v4-v5V4 region of the *16S rRNA* gene on the '*Ca.* P. solani' infected and non-infected group produced, after filtering out organellar sequences and rare OTUs, a total of 527466 sequences belonging to 363 different OTUs. Out of all the obtained sequences, 228190 belong to '*Ca.* P.solani' infected group and 299276 to the non-infected group. Number of sequences and OTUs obtained from the '*Ca*. P. solani' infected and non-infected group are reported in Table 2.

The alpha diversity indices of Observed, Chao1 and Shannon were used for this study as shown in Fig. 1. The observed OTUs were considered to show the absolute richness. The values of this parameter range from a minimum average of 17, found in non-infected *E. incisus* and *D. europaea*, to a maximum of 106, found in infected *E. incisus*. The corrected estimation of richness made through the Chao1 index are very close to the value of Observed for most samples, indicating that the sequencing has reached an adequate depth, having very few singletons and a low number of estimated undetected OTUs. The Shannon index, indicating the evenness of species distribution ranges from a minimum of 0.089 in non-infected *E. variegatus*, to a maximum of 2.25 in infected *E. incisus*. For *E. incisus, E. variegatus* and *H. obsoletus*, the number of Observed OTU and the Shannon index are significantly different between infected and non-infected samples, indicating that the presence of the phytoplasma has a strong effect on the alpha-diversity of the bacterial community in these species. For all other considered insect species, no statistically significant difference was found between these values for infected and non-infected groups. The bacterial distribution of the different insect species both infected and non-infected groups were characterized in terms of the relative taxonomic abundance. A total of 18 phyla, 46 classes, 58 orders, 89 families, 100 genera and 35 species (of which a total of 277 with an unidentified taxa) ~~(Fig. 2)~~.

Core microbiome

In order to highlight the existence of an identifiable common core microbiome, the group of members shared among the microbial community of the infected and non-infected groups of the different insect species were identified. The bacterial communities in these insect populations are clearly distinct and do not share a common core as no single OTU is shared (i) among individuals of all insect species regardless of infection, (ii) among infected individuals regardless of insect species, (iii) among non-infected individuals regardless of insect species.Venn diagrams were used to represent the number of OTUs found exclusively in the infected group, non-infected group, or shared between the two groups (Fig. 2). For most of the analyzed species, a common trend can be identified with infected individuals showing a much higher number of unique OTUs compared to non-infected samples. This difference is particularly pronounced in *E. incisus* and *E. variegatus* (Fig. 2 and 3). This is true for all species, except *D. europaea* and *P. spumarius*, for which the number of unique OTUs in infected and non-infected samples is very similar (Fig. 2). Interestingly, regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65 shared OTUs (Fig. 2).

Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between infected and non-infected in all species. Other relevant bacterial genera that are core between infected and non-infected in particular species are *Cronobacter* and *Sodalis* (*C. viridis*), *Erwinia* (*P. spumarius*), *Propionibacterium* (*E. variegatus*), *Purcelliella* (*H. obsoletus*), and *Rickettsia* (*H. obsoletus*).

Bacterial community structure

Venn diagram representation showed a qualitative difference among OTUs identified in infected and non-infected individuals in the species, without considering the vital quantitative aspect in describing the community structure. To compare the microbial community structure among the '*Ca.* P. solani' infected and non-infected individuals within and among insect species, principle coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 4).

The graph shows that the species is a major driver of diversity among the microbial communities, as each species tends to form a separate cluster. From this analysis, two groups of insects can be identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on species alone, with the single samples of infected and non-infected insects overlapping and mixing with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct clusters based on species for non-infected samples, but the infected samples do form clusters based on species, distinct from the non-infected samples within the same species. These results were confirmed by an Adonis multivariate analysis of variance, showing that there are statistically significant differences between the structure of the community in infected and non-infected samples of *E. incisius* (p=0.001), *E. variegatus* (p=0.013) and *P. alienus/confinis* (p=0.006), while no significant differences were found in the other four species.

Bacterial abundance and distribution

The composition in taxa of the microbial communities according to the different insect species as well as the different infection status are reported in the bar plots in Fig. 5~~3~~. All detected OTUs could be assigned to one of ten phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Proteobacteria, or Tenericutes (Fig. 5~~3~~A). In most analyzed samples, the most abundant phylum is Bacteroidetes, which can compose up to 99% of the total community, as for the non-infected *E. variegatus*. This dominance of Bacteroidetes is seen in all non-infected samples, except for *H. obsoletus*, and also in some infected insect species: *D. europaea*, *P. spumarius*, and *P. alienus/confinis*. The second most abundant

phylum in most samples is Proteobacteria: this phylum is the most abundant in *H. obsoletus*, both infected and non-infected, and is also highly abundant also in *C. viridis*, *D. europaea*, and *P. spumarius*, both in infected and non-infected samples. While mostly absent in non-infected samples, the phyla Actinobacteria and Firmicutes are found with higher abundance in the infected samples of *E. incisus*, and *E. variegatus*. ~~As expected, the phylum Tenericutes, which includes '*Ca.* P. solani', is found with high abundance only in the infected samples and, even then, only in three species: *E. incisus*, *E. variegatus*, and *P. alienus/confinis*.~~ Bacteria belonging to the phylum Cyanobacteria are found only in the infected samples of *E. incisus* and *E. variegatus*.

In most of the examined insect species, the microbial community was composed of members of few genera, but at very high abundance. In fact, ~~A~~as it can be seen by comparison of Fig. 5~~3~~A and 5~~3~~B, almost the entire abundance of the Bacteroidetes phylum can be ascribed to the genus *Sulcia* alone. Likewise, the Tenericutes abundance is due uniquely to the presence of OTUs of '*Ca.* Phytoplasma' and, in *H. obsoletus*, the abundance of Proteobacteria overlaps with the abundance of *Purcelliella*. In contrast, ~~In~~ the microbiota of *E. incisus* ~~there are~~shows many more genera, but the ~~twelve~~ 12 most abundant ones only cover 60% of the abundance, while the rest are less abundant genera. Regarding '*Ca*. Phytoplasma' OTUs, they are found exclusively in infected individuals of all species, but with high abundance (>1%) only in *E. incisus*, *E. variegatus*, and *P. alienus/confinis* (Table 3).

Comparing the infected and non-infected abundance of different genera, it emerges that for some species there are no changes, or very little changes, in the structure of the bacterial community in the presence of absence of '*Ca.* Phytoplasma solani': *C. viridis*, *D. europaea*, and *P. spumarius* (Fig. 5~~3~~B). For the main host, *H. obsoletus*, the community itself does not seem to undergo great variations in quality, with the addition of only *Rickettsia* in infected samples, but the relative abundance of the members of the community are vastly different. Similarly, for *P. alienus/confinis* the community only shows the addition of '*Ca*. Phytoplasma' between healthy and infected samples, but the abundance of OTUs belonging to this genus is very high, suggesting a strong interaction between this plant pathogen, the host, and the microbial community already present in the host. For the remaining examined species (*E. incisus*, *E. variegatus*) the infection by the phytoplasma is accompanied by a radical change in the microbial community (Fig. 5B4).

Regarding the bacterial genera that were of particular interest in this study, it can be seen that (i) the '*Ca.* Phytoplasma'-antagonistic *Dyella* is not found in any of the examined insect species.~~,~~ (ii) ~~the possibly insect-antagonistic~~ *Wolbachia* is found in non-infected specimens of all vector species, but with high abundance (>1%) only in ~~-~~*D. europaea*, *E. incisus*, *H. obsoletus*, and *P. spumarius*; in all examined vector species, with the exception of *D. europaeaeae*~~ae~~, the abundance

of this genus is reduced in the infected samples, to the degree of disappearing entirely from the community for *E. incisus* and ~~*P. spumarius*~~*E. variegatus*. *Wolbachia* is not found in *C. viridis* regardless of phytoplasma infection (Table 3). (iii) The mutualistic symbiont *Sulcia* makes up for ~~a very relevant part~~the majority of the microbiota in non-infected specimens of all insects, except *H. obsoletus*. Within phloem-feeders, it showed an abundance >95% in *E. incisus*, *E. variegatus* and *P. alienus/confinis*, ~75% in *D. europaea*, and ~30% in *H. obsoletus*. Within xylem-feeders (*C. viridis* and *P. spumarius*), it showed an abundance <75%. ~~of these sap-feeding insects and, w~~With the exception of the xylem-feeders and ~~of *C. viridis*, *D. europeae*~~*europaea*~~, and *P. spumarius*~~, its abundance is greatly reduced in infected samples, compared to non-infected samples of the same species (Table 3).

~~**Bacterial community structure**~~

~~To gain insight into similarities in the bacterial community structure among the '*Ca.* P. solani' infected and non-infected insect species, principle coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis dissimilarity (Fig. 5). The graph shows that the species is a major driver of diversity among the microbial communities, as each species tends to form a separate cluster. From this analysis, two groups of insects can be identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on species alone, with the single samples of infected and non-infected insects overlapping and mixing with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct clusters based on species for non-infected samples, but the infected samples do form clusters based on species, distinct from the non-infected samples within the same species. These results were confirmed by an Adonis multivariate analysis of variance, showing that there are statistically significant differences between the structure of the community in infected and non-infected samples of *E. incisius* (p=0.001), *E. variegatus* (p=0.013) and *P. alienus/confinis* (p=0.006), while no significant differences were found in the other four species.~~

~~**Core microbiome**~~

~~In order to highlight the existence of an identifiable common core microbiome, the group of members shared among the microbial community of the infected and non-infected groups of the different insect species were identified. Venn diagrams were used to represent the number of OTUs found exclusively in the infected group, non-infected group, or shared between the two groups (Fig. 6). For most of the analyzed species, a common trend can be identified with infected individuals showing a much higher number of unique OTUs compared to non-infected samples. This is true for~~

~~all species, except *D. europaea* and *P. spumarius*, for which the number of unique OTUs in infected and non-infected samples is very similar. Interestingly, regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65 shared OTUs. No single OTU is shared among all species, nor among all infected or non-infected samples, indicating that the bacterial communities in these populations are clearly distinct and do not share a common core.~~

~~Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between infected and non-infected in all species. Other relevant bacterial genera that are core between infected and non-infected in particular species are *Cronobacter* (*C. viridis*), *Erwinia* (*P. spumarius*), *Propionibacterium* (*E. variegatus*), *Purcelliella* (*H. obsoletus*), *Rickettsia* (*H. obsoletus*), and *Sodalis* (*C. viridis*).~~

DISCUSSION

~~Until recently, *H. obsoletus* and *R. panzeri* were believed to be the only insect vectors of '*Ca*. P. solani' to grapevine, but recent researches allowed the identification of several new insect vectors (Quaglino *et al.* 2019).~~ The insect survey and molecular identification of '*Ca*. P. solani', conducted in this study, confirmed the presence of abundant populations and the unusually high infection rate (>10%) in 2018 for the main vector *H. obsoletus* and for a majority of the insect species ~~newly~~ recently reported as~~insect~~ vectors~~species~~ (Quaglino *et al.* 2019). If the scenario of containing Bois noir disease in vineyards was already bleak due to the high polyphagia of the established insect vectors, the addition of several more vectors is leading to the idea that there are no options to implement any traditional containment strategy against this disease, its pathogen, or vectors. A comprehensive and thorough investigation of the bacterial diversity in '*Ca*. P. solani' insect vectors is essential for understanding how this pathogen interacts with its hosts and their microbiota, possibly leading to the development of effective prevention and treatment strategies based on the management of the bacterial community in the vectors.

This study analyzes the bacterial community present in insects associated to '*Ca.* P. solani' collected in vineyards in northern Italy, including the main vector *H. obsoletus*, five newly reported vector species (*D. europaea, E. incisus*, *E. variegatus*, *P. spumarius*, and *P. alienus*/*confinis*) and a species that is known to host the phytoplasma but not to transmit it, *C. viridis*. In addition to investigating and describing the bacterial community found in these insects, both when they're infected with '*Ca*. P. solani' and when they aren't, the study focuses on the presence of specific genera of bacteria that have been reported as potentially essential for the survival of the insect

(genus *Sulcia*), as potential parasites of the vectors (genus *Wolbachia*), or as antagonistic towards the phytoplasma (genus *Wolbachia* and *Dyella*).

In comparison with previous studies on the topic of the bacterial communities associated to insect vectors of '*Ca.* P. solani', this study uses a more modern technique than those previously employed [LH-PCR, DGGE (Gonella *et al*. 2011); sequencing with Roche 454 (Iasur-Kruh *et al*. 2017)]) and extends the range of investigation to more vectors, instead of analyzing just *H. obsoletus*.

~~The results obtained from the investigation of the bacterial microbiota of these insect species allows the formulation of several considerations.~~

Starting from the parameters of alpha-diversity, it is found that in these insects the microbial communities do not have a high diversity, showing a low number of different OTUs that dominate the whole community. This is particularly true for the non-infected samples that showed less than 20 different OTUs for most of the analyzed species. This result is in accordance with what was previously presented regarding the bacterial communities of phloem-/xylem-feeding insects, and it is hypothesized that this is due to their extremely specialized diet which (i) requires specific metabolic processes to implement the insect's own and ensure survival and (ii) comes from a compartment of the plant that is colonized only by very specialized bacteria and therefore acts as a low-diversity reservoir from which the insects ingest bacteria (Colman *et al.* 2012; Jing *et al.* 2014; Overholt *et al.* 2015).

For most species there is no difference in the alpha-diversity parameters between '*Ca.* P. solani' infected and non-infected specimens, indicating that the presence of the pathogen does not lead to a major change in the qualitative composition of the community. Still, for *E. incisus* and *E. variegatus* a statistically significant increase was observed for all parameters in the infected specimens, compared to the non-infected.

The analysis of abundance of the different taxa in the insect species in general revealed microbial communities with low diversity, in which only a handful of genera were present with high abundance: *Bacillus* (Firmicutes), '*Candidatus* Phytoplasma' (Tenericutes), *Cronobacter* (Proteobacteria), *Erwinia* (Proteobacteria), *Propionibacterium* (Actinobacteria), *Purcelliella* (Proteobacteria), *Rickettsia* (Proteobacteria), *Sodalis* (Proteobacteria), *Staphylococcus* (Firmicutes), *Sulcia* (Bacteroidetes), and *Wolbachia* (Proteobacteria).

The results obtained on the description of the bacterial microbiota of *E. incisus* and *P. alienus/confinis* agree with what was previously reported by Kobialka *et al.* (2018), who indicated a microbial community dominated by *Sulcia* for these species.

Regarding *H. obsoletus*, our results that highlighted the presence of the genera *Sulcia*, *Wolbachia*, and *Purcelliella* confirming data ~~the results~~ previously obtained by Bressan *et al.* (2009) and Gonella *et al.* (2011) in northern Italy, but not ~~with~~ those obtained by Iasur-Kruh *et al.* (2017) in Israel. This latter study determined, using both classical and molecular microbiology methods, that the genus *Sulcia* was the most abundant in *H. obsoletus*, followed by *Pectobacterium*. These differences may be explained by several variables, such as the different techniques used, and the different geographical areas from which specimens were sampled ~~(northern Italy and Israel)~~, which leads to different climatic conditions and insect diet.

The results obtained on *C. viridis*, with a high abundance of the genera *Sulcia* and *Sodalis* are in accordance to those previously reported by Michalik *et al.* (2014). Intriguingly, t~~T~~hese results also revealed that *C. viridis* has a unique microbiota compared to the other insect species analyzed: it has a more diverse composition, in which five different genera have a relevant level of abundance~~,~~ and it's the only species in which we find a high abundance of the genera *Cronobacter* and *Sodalis*. These results suggest that either the higher diversity, leading to a more resilient bacterial community, or these specific genera of bacteria could play a role in determining the non-vector status of this insect. Further studies will be conducted to determine if these elements can indeed be important and relevant for the development of an MRM strategy to reduce the spread of Bois noir.

The results regarding the beta-diversity in each analyzed insect species, infected and non-infected, highlighted the presence of two different groups among the insect species: (i) insects for which the presence or absence of the phytoplasma did not cause a major restructuring of the bacterial community, including the species *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius*; and (ii) insects for which the presence of the phytoplasma, not related to its abundance, caused a major change in the bacterial community, including the species *E. incisus*, *E. variegatus*, and *P. alienus/confinis*. Interestingly, among the analyzed species, these three are the only ones belonging to the subfamily Deltocephalinae. The microbiota associated with members of this subfamily is usually characterized by the presence of two ancient mutualistic endosymbiotic bacterial genera: *Sulcia* and *Nasuia* (Kobiałka *et al*. 2018). However, several studies reported that the symbiotic systems of Deltocephalinae leafhoppers can be very diverse, driven by processes of symbiont acquisition and replacement, which can include both bacteria and fungi (Nishino *et al*. 2016; Brentassi *et al*. 2017; Kobiałka *et al*. 2018; Mao and Bennett 2020). In our datasets, no OTUs assigned to the genus *Nasuia* were detected. This result is not in accordance with what is reported by Kobiałka *et al*. (2018), which found *Nasuia* in *E. incisus* and *P. alienus/confinis*. On the other hand, a similar situation, in which *Nasuia* was not detected and *Sulcia* represented more than 95%

of microbiota OTUs, was reported in *Dalbulus maidis* (subfamily Deltocephalinae) (Brentassi *et al.* 2017). *D. maidis* is the vector of '*Ca.* Phytoplasma asteris' (Raygoza and Nault 1998), associated with maize bushy stunt disease, a phytoplasma strictly related to '*Ca.* P. solani' (Quaglino *et al.* 2013). Considering these data, it is reasonable to propose that the symbiotic systems in our insect populations are prevalently based on *Sulcia*. Furthermore, in North Italian vineyards, *Nasuia* was not identified in *Scaphoideus titanus* (subfamily Deltocephalinae), the insect vector of the phytoplasma associated with flavescence dorée disease of grapevine (Sacchi *et al.* 2008). This could suggest the hypothesis that, in North Italy, the environmental conditions of vineyard agroecosystems do not favor *Nasuia* as mutualistic endosymbiont of phytoplasma insect vectors.

For the species *C. viridis*, *D. europaea*, *H. obsoletus* and *P. spumarius*, our results are in accordance with what was reported by Fagen *et al.* (2012) regarding the bacterial community of *Diaphorina citri*, the vector of another obligate plant pathogen '*Ca.* Liberibacter asiaticus': the microbiota of these insects was dominated by the same three or four genera regardless of the presence or abundance of the plant pathogen. ~~But, a~~As the presence of the phytoplasma does affect the microbial community in the other three analyzed species, it becomes evident that it i~~'~~s not the presence of phytoplasma ~~alone~~ that determines a change in the microbial community, but ~~rather an~~the interaction between phytoplasma, insect host, and bacterial community. As expected from their common feeding behavior, the xylem-feeding species *C. viridis* and *P. spumarius* showed a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. ~~Anyway~~However, the aforementioned unicity of *C. viridis* microbiota is not due exclusively by its source diet, which is shared by *P. spumarius*~~.~~ ~~This~~ reinforcinges ~~the~~ the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Regarding the specific genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), interesting considerations can be made for *Sulcia* and *Wolbachia*, while *Dyella* was not found to be present in any of the analyzed specimens. This might be due to the time of sampling, as it was reported that the presence of *Dyella*-like bacteria increases in the late stage of the season (Iasur-Kruh *et al.* 2017).

In terms of abundance, the genus *Sulcia* was found to be the most abundant in all non-infected insect species except *H. obsoletus* where it was the second most abundant after *Purcelliella*. This result is in agreement with Moran *et al.* (2005) who showed that several Auchenorrhyncha insect lineages, including Cicadomorpha and Fulgomorpha, house a single phylotype bacterium called '*Candidatus* Sulcia muelleri'. In the infected groups there was a dramatic decrease in the genus *Sulcia*; except in the case of *C. viridis*, *D. europaea*, and *P. spumarius* where the reduction was quite low.~~-~~ This reduction in the abundance of *Sulcia* has

several possible explanations: the first is that the interaction between the phytoplasma and other members of the microbiota lead to a rise of secondary mutualists, disadvantaging the primary mutualists such as *Sulcia* (Heddi *et al.* 1998); a second hypothesis is related to the host's immune response: it was demonstrated by Galetto *et al.* (2018) that the insect, *E. variegatus* in that study, can activate a strong immune response when interacting with a phytoplasma that is not the one that it usually transmits. This immune reaction could change the bacterial community inside the host drastically, favoring more resistant bacteria, in particular Gram-positive species, as is seen in our study for *E. incisus* and *E. variegatus*. A third hypothesis is based on results obtained of *D. citri* and '*Candidatus* Liberibacter asiaticus' by Vyas *et al*. (2015): this study demonstrated that the phytopathogen could modulate free amino acids availability by interfering with hexamerin storage pathways by regulating expression of amino acid storage protein genes. Such evidence suggests that the reason why there is a dramatic reduction in genus *Sulcia*, which is heavily committed to amino acid production and encodes enzymes for synthesis of all amino acids required as animal nutrients, is simply due to the fact that an infected insect does not need such a high abundance of this bacterial genus. On the other hand, sap-feeding insects rely heavily on the contribution of their obligate symbionts to maintain their metabolism (McCutcheon and Moran 2007). For this reason, the loss of dominance by the beneficial *Sulcia* endosymbionts could instead prove to be detrimental to the insect's fitness. More data on the fitness of the infected and non-infected insects would be needed to give a correct interpretation of this result.

Genus *Wolbachia* tended to be present only in the non-infected specimens and was ~~completely eliminated~~largely reduced in the infected insect species, except in the case of ~~(i) *H. obsoletus*, in which it was still present but with lower abundance; and (ii)~~ *D. europaea*, in which the abundance of *Wolbachia* was higher in the '*Ca*. P. solani' infected group ~~than the non-infected group~~. ~~Similar results were obtained by Fagen *et al.* (2012) who reported that the '*Ca.* Liberibacter asiaticus' titer within the insect was found to have a strong positive relationship with *Wolbachia* endosymbiont. Also, in this case,~~From these results, it becomes evident that the interaction is not just between the phytoplasma and *Wolbachia*, but that the insect species and the rest of the microbiota play a role in determining ~~whether the abundance of *Wolbachia* is increased or reduced upon infection~~its outcome. Still, considering that co-presence of phytoplasma and *Wolbachia* was not observed in the majority of the insect species, in general it is reasonable to conclude that a negative interaction governs the relationship between phytoplasma and *Wolbachia*. It should be established whether phytoplasma infection affects the *Wolbachia* concentration or if the presence of *Wolbachia* confers protection either by reduction in pathogen load, or competition with the pathogen (Krstić *et al*. 2018).

~~The general reduction in the abundance of *Wolbachia* in phytoplasma-infected specimens suggests that the phytoplasma can protect the insect from the possible detrimental effects of this intracellular bacterium (feminization, male killing and a sperm-egg incompatibility) and might make *Wolbachia* an unsuitable target for the development of an MRM strategy.~~

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CONCLUSION

This study described the bacterial communities associated with seven insect species hosting '*Ca*. P. solani' and found in vineyards in North Italy. The mutualistic endosymbiont *Sulcia* was found as the prevalent member of the microbiota in all insect individuals non-infected by the phytoplasma. The non-vector *C. viridis* carries unique bacterial signatures (i.e, *Sodalis*, *Cronobacter*) distinguishing its microbiota from that of vector insects, including its fellow xylem-feeder *P. spumarius*.

Beta-diversity analysis revealed that the xylem-feeding behavior of *C. viridis* and *P. spumarius* gave a more similar structure in their microbiota in comparison with those of the phloem-feeding vectors. Anyway, the aforementioned unicity of *C. viridis* reinforces the hypothesis that microbiota elements could influence the vector / non-vector status of phytoplasma host insects.

Analyses highlighted that, in North Italy, phytoplasma infection (not related to its abundance) is associated with major change due to an increase of diversity in the microbiota structure exclusively in *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, the only species, among the analyzed ones, belonging to the subfamily Deltocephalinae.

Considering the specific bacterial genera on which our study focused (*Sulcia*, *Wolbachia*, and *Dyella*), obtained data showed that '*Ca*. P. solani' may have an adverse effect on the presence of ~~the obligate endosymbiont '*Ca. Sulcia* muelleri'~~ as well as ~~the facultative endosymbiont~~ *Wolbachia*, while *Dyella* was not found. Further studies are necessary to elucidate whether observed differences (reduction of *Sulcia* and *Wolbachia*, and increase of bacterial diversity) in phytoplasma infected insects are associated with fitness increase or decrease.

The results of this study indicate an interesting perspective regarding the microbial signatures that could be relevant to determine whether an insect species can be a vector or not, opening up new avenues for developing MRM-based approaches to contain BN spreading.

~~‘*Ca*. P. solani’ may have an adverse effect on the presence of the obligate endosymbiont ‘*Ca*. Sulcia muelleri’ as well as the facultative endosymbiont *Wolbachia*.~~

~~Such reduction in the abundance levels of ‘*Ca*. Sulcia muelleri’ in the ‘*Ca*. P.solani’ infected insect vectors may be a marker of the increased fitness of the insect, indicating that the host becomes more efficient in utilizing its metabolic resources, since *Sulcia* is responsible for critical nutritional procedures inside the host. The presence of *Wolbachia* might down-size the population of the different insect vectors due to its biological control activity, making it a prime candidate for biocontrol of these vectors. Still, as these bacteria are reduced, or even eliminated, in the ‘*Ca*. P. solani’-infected specimens, it may be hard to control the populations of vectors using *Wolbachia* as a biological control agent.~~

~~The results of this study, describing the differences in the bacterial communities between six ‘*Ca*. P. solani’-vector and one non-vector species (*C. viridis*), and highlighting several differences between them indicate an interesting perspective regarding the microbial determinants that could be relevant to determine whether an insect species can be a vector or not, opening up new avenues for developing MRM-based approaches to contain BN spreading.~~

FUNDING

This work was partially funded by Consorzio per la Tutela del Franciacorta.

CONFLICT OF INTEREST

None declared.

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