

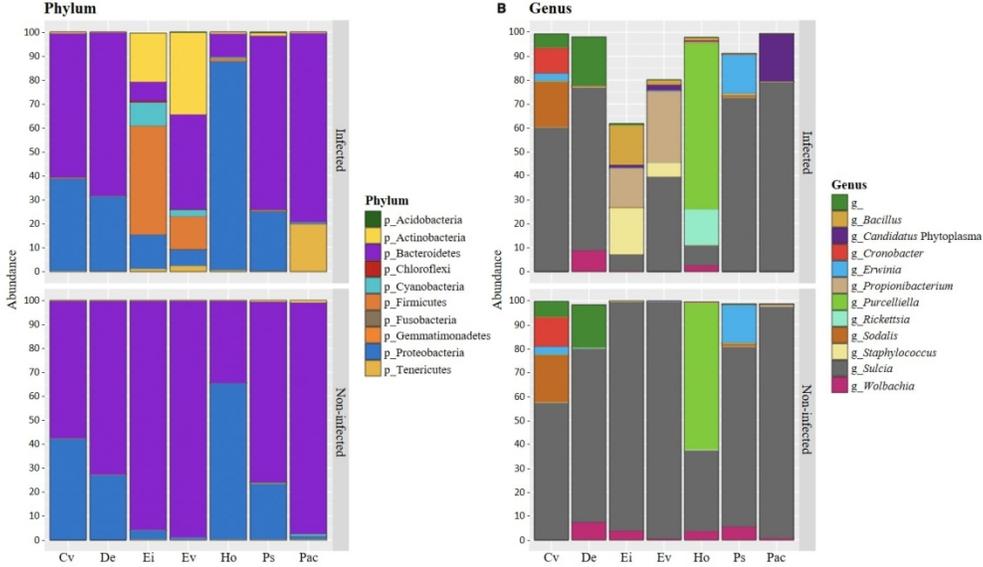
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Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in relation to phytoplasma infection

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Keywords:	grapevine yellows, <i>Wolbachia</i> , <i>Sulcia</i> , microbial resource management, phloem-limited bacteria

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1 Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in 2 relation to phytoplasma infection

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

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22 23 **ABSTRACT**

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

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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*,

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3 70 *Laodelphax striatella*, *Phylloxera spumarius*, and *Psammotettix alienus/confinis* (Quaglino *et al.*
4 71 2019).

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6 72 Since the cycle includes so many highly polyphagous insects and a very broad range of
7 73 secondary wild hosts, it is difficult to organize effective prevention and containment measures
8 74 (Bertaccini *et al.* 2014; Moussa *et al.* 2019; Quaglino *et al.* 2019). Moreover, the typical
9 75 management strategies for phytoplasma diseases, based on the control of the vector with
10 76 insecticides and the removal of infected plants (Bianco *et al.* 2011), are not effective against ‘*Ca. P.*
11 77 *solani*’ (Angelini *et al.* 2018). For this reason, other methods are being envisioned, including the use
12 78 of Microbial Resource Management (MRM).

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14 79 MRM is the proper management of the microbial resources available in a given ecosystem
15 80 in order to solve a practical problem by directing the potential of microorganisms. In particular, on
16 81 the topic of control of insect vectors, some first steps have already been taken towards defining the
17 82 composition and functionality of microbial communities associated with insects (Marzorati *et al.*
18 83 2006; Miller *et al.* 2006; Crotti *et al.* 2012).

19 84 Insects, like all other animals, maintain several symbiotic interactions with their associated
20 85 microbial community, which has a great influence on their fitness, evolution, and diversity
21 86 (Margulis and Fester 1991; Ruby *et al.* 2004). The microbial community can contain beneficial
22 87 symbionts, called mutualists, but also detrimental ones, which are parasites or pathogens, and the
23 88 dynamic balance found in a microbial community can produce either a positive or negative effect
24 89 for the health of the host (Berg *et al.* 2014; Lebeis 2014). An MRM approach to control insect
25 90 vectors would therefore be performed by manipulating their microbial community to promote the
26 91 effect of naturally present antagonistic microorganisms (Trivedi *et al.* 2016).

27 92 A negative prospect for this strategy is that, as the interactions between environment, host,
28 93 and microbiota are very complex and influenced by several variables (Trivedi *et al.* 2015; Douglas
29 94 2015; Fonseca-García *et al.* 2016), more studies need to be conducted in the description of the
30 95 bacterial community associated to these vectors before its manipulation can become a viable option.
31 96 The positive prospect, since these phloem-feeding insects rely heavily on obligate bacterial
32 97 symbionts to provide nutrients which are lacking in their unbalanced diet (Buchner 1965; Baumann
33 98 2005; Bourtzis and Miller 2006; Skidmore and Hansen 2017), is that these insects will be
34 99 particularly susceptible to unbalances in their microbial community.

35 100 A main actor in these obligate mutualistic interactions is ‘*Candidatus Sulcia muelleri*’, a
36 101 bacterial species that greatly reduced its genome as it evolved as an obligate symbiont; moreover, it
37 102 is documented to be strictly associated to leafhoppers and planthoppers, among other hosts (Moran
38 103 *et al.* 2005; McCutcheon *et al.* 2009). This bacterial species is involved in the synthesis of several

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3 104 amino acids necessary for the insect host (McCutcheon and Moran 2007). Other mutualistic
4 105 bacteria involved in these interactions belong to the genera *Nasuia* and *Sodalis* (Kobialka *et al.*
5 106 2018).

8 107 Another bacterial genus interesting for MRM approach is *Wolbachia*, ubiquitous
9 108 endosymbionts associated with over 60% of known insect species, as well as other arthropods and
10 109 nematodes (Hosokawa *et al.* 2010; Zug and Hammerstein 2012; Newton and Rice 2020).
13 110 *Wolbachia* species are cytoplasmically inherited and known as reproductive parasites due to their
15 111 ability to manipulate reproduction such as sperm-egg incompatibility (cytoplasmic incompatibility),
16 112 parthenogenesis induction, male killing, and feminization, making it a possible biocontrol agent
17 113 against the vectors (Werren 1997; Stouthamer *et al.* 1999; Werren *et al.* 2008; Brelsfoard and
20 114 Dobson 2009; Chuche *et al.* 2017). Nevertheless, several studies showed that *Wolbachia* can act as
22 115 mutualistic towards insect hosts, modulating nutrition and immune responses (Hosokawa *et al.*
23 116 2010; Iturbe-Ormaetxe *et al.* 2011; Newton and Rice 2020). Moreover, recent studies proposed that
25 117 *Wolbachia* can act as biocontrol agent of insect-transmitted pathogens, including phytoplasmas, by
26 118 increasing latency period and blocking pathogen transmission (Shaw *et al.* 2016; Chuche *et al.*
28 119 2017).

31 120 *Dyella*-like bacterium (DLB), gram-negative, aerobic, rod-shaped endophytic bacteria
32 121 belonging to the family Rhodanobacteraceae, can be acquired by feeding and has shown a potential
34 122 biocontrol activity against phytoplasmas and their cultivable relative *Spiroplasma melliferum*
35 123 (Iasur-Kruh *et al.* 2017, 2018). The possible mechanisms of DLB antagonism towards
37 124 phytoplasmas have been hypothesized to be (i) competition for nutrients or colonization niches, (ii)
39 125 induction of plant systemic resistance, (iii) secretion of plant growth hormones, or (iv) secretion of
40 126 phytoplasma growth inhibitory substances (Eljounaidi *et al.* 2016).

43 127 In this scenario, the current study aims to characterize through an Next Generation
44 128 Sequencing (NGS) approach the bacterial community associated with selected '*Ca. P. solani*' insect
46 129 hosts, both infected and non-infected by the phytoplasma, with the following goals: (i) describe the
47 130 bacterial communities in different insect hosts of '*Ca. P. solani*'; (ii) determine whether the
49 131 presence of '*Ca. P. solani*' affects the bacterial community, in particular if it can cause a
51 132 dysbiosis (also called dysbacteriosis) or increase diversity; (iii) evaluate the presence of possible
53 133 antagonists towards the insect (e.g. *Wolbachia* spp.) or phytoplasma (e.g. *Wolbachia* spp. and
54 134 *Dyella*-like bacteria); (iv) investigate the effect of '*Ca. P. solani*' on the obligate endosymbiont '*Ca.*
56 135 *Sulcia*' spp.. The selected insects are the main vector *H. obsoletus*, newly reported vectors (phloem-
58 136 feeders: *A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, and *P.*
59 137 *alienus/confinis*; xylem-feeder: *P. spumarius*), and *Cicadella viridis*, one of the most abundant

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3 138 insects living in Italian vineyard, harboring with high infection rate but not vectoring ‘*Ca. P. solani*’
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5 139 (Quaglino *et al.* 2019), and characterized by xylem-feeding activity. *C. viridis* was included in the
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7 140 study for comparing the microbiota associated with xylem- and phloem-feeders, and investigating
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9 141 the phytoplasma influence on the microbiota structure in comparison with vectors. *R. panzeri* was
10 142 not among the selected vectors because it is not found in the studied area.

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12 143 Achieving the previously mentioned aims regarding the description of the bacterial
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14 144 communities may help in devising MRM-based approaches to achieve the main objective of
15 145 biological control of ‘*Ca. P. solani*’ and its insect vectors.
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19 147 MATERIALS AND METHODS

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22 149 Insect collection

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24 150 Specimens of the insect species *A. makarovi*, *C. viridis*, *D. hamata*, *D. europaea*, *E. incisus*, *E.*
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26 151 *variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius*, and *P. alienus/confinis* were captured by
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28 152 sweep entomological net in mid-July 2018 in the Chardonnay organic vineyard (Franciacorta,
29 153 Lombardy Region, North Italy; N 45°35'38.12", E 10°09'34.32") where new insect vectors of ‘*Ca.*
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31 154 *P. solani*’ had previously been identified (Quaglino *et al.* 2019). Insect individuals were stored in
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33 155 ethanol 90%, transferred to the lab for species identity confirmation by stereomicroscope based on
34 156 the taxonomic keys of den Bieman *et al.* (2011), and maintained in absolute ethanol at 4°C till use.
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36 157 Regarding the genus *Psammotettix*, given that the dichotomous keys are related only to males, the
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38 158 species *P. alienus* and *P. confinis* were considered together.
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41 160 Total nucleic acids extraction and suitability for amplification

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43 161 Total nucleic acids (TNAs) were extracted from ethanol preserved insects (dried by filter paper)
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45 162 through homogenization in a CTAB-based buffer [2% w/v cetyltrimethylammonium-bromide
46 163 (CTAB); 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HCl pH 8.0; 0.5% ascorbic acid]. After
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48 164 incubation at 60°C for 20 min, TNAs were separated with one volume of chloroform: isoamyl
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50 165 alcohol 24:1 v/v solution and precipitated with the addition of one volume of cold isopropanol. The
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52 166 TNAs pellet was then washed with ethanol 70%, air dried, dissolved in 30µL of TE buffer pH 8.0,
53 167 and maintained at –20 °C until use (Moussa *et al.* 2019).
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55 168 The suitability of the extracted TNAs for amplification was tested through a bacterial *16S*
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57 169 *rRNA* gene PCR assay using the universal primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-
58 170 3') / 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). PCR reactions were
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60 171 conducted in Applied Biosystems 2720 thermocycler (Applied Biosystems, Monza, Milan) with the

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3 172 following conditions: 2 min at 95 °C; 35 cycles consisting of 1 min at 95 °C, 1 min 30 s at 50 °C
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5 173 and 2 min at 72 °C; 10 min at 72 °C. PCR reactions were performed in 25 µL volume containing 50
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7 174 µM of each dNTP, 0.4 µM of each primer, 1.5 mM MgCl₂, 1× polymerase buffer, 1 unit GoTaq
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9 175 polymerase enzyme (Promega, Milan, Italy). PCR mixture devoid of TNAs was employed as
10 176 negative control. PCR products were analyzed by electrophoreses in 1% agarose gel stained with
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12 177 Midori green under a UV transilluminator. Only the samples that gave positive amplification with
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14 178 this reaction were considered for further analyses.

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17 180 **Molecular detection of ‘*Candidatus Phytoplasma solani*’**

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19 181 The presence of ‘*Ca. P. solani*’ in collected insects was verified by species-specific nested PCR-
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21 182 based amplification of the *stamp* gene using the primer pair *Stamp-F* (5’-
22 183 GTAGGTTTTGGATGTTTTAAG-3’) / *Stamp-R0* (5’-AAATAAAAGAACAAGTATAGACGA-
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24 184 3’), followed by the primer pair *Stamp-F1* (5’-TTCTTTAAACACACCAAGAC-3’) / *Stamp-R1* (5’-
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26 185 AAGCCAGAATTTAATCTAGC-3’) (Fabre *et al.* 2011). PCR reactions were conducted in
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28 186 Applied Biosystems 2720 thermocycler with the following conditions: 4 min at 94 °C; 35 cycles
29 187 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
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31 188 °C; 7 min at 72 °C. PCR mixture devoid of TNAs was employed as negative control. PCR reaction
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33 189 mixtures and PCR products visualization were as described above for bacterial *16S rRNA* gene.

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36 191 **Illumina Mi Seq sequencing**

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38 192 Based on the molecular detection of ‘*Ca. P. solani*’ and the requested TNAs quantity (at least 0.5
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40 193 µg) / quality (ratio 260/280 nm ~2), TNAs extracted from 96 insect specimens were selected to
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42 194 undergo Illumina Mi Seq sequencing. These 96 samples were picked to ensure that at least five
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44 195 samples for each insect species were included in both the ‘*Ca. P. solani*’-infected and non-infected
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46 196 groups.

47 197 Next generation sequencing library preparations and Illumina Mi Seq sequencing were
48 198 conducted by an external provider (Personal Genomics, Verona, Italy). The bacterial *16S rRNA*
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50 199 gene hypervariable region V4 libraries were prepared using the forward primer 515FB (5’-
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52 200 GTGYCAGCMGCCGCGGTAA-3’) and 806RB (5’-GGACTACNVGGGTWTCTAAT-3’), and the
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54 201 amplification of sequences belonging to mitochondria was blocked using a PNA blocker (Lundberg
55 202 *et al.* 2013). Metagenomic sequencing was performed using the Illumina Miseq 300PE sequencing
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57 203 technology. Obtained reads were deposited in the EMBL-ENA under the project number
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59 204 PRJEB38750.

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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 Shannon 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

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Insects collected and ‘*Ca. P. solani*’ infection rate. A total of 400 individuals were captured. The

4 most abundant species were *E. variegatus* (75 individuals), *P. alienus/confinis* (71) and *E. incisus*

5 241 (59), while *L. striatella* (16), *D. hamata* (8) and *A. makarovi* (6) were scarcely present (Table 1).

6 242 Bacterial 16S rDNA fragment 27F/1492R was amplified by the TNAs extracted from all insect

7 243 specimens and not in the negative control, evidencing the TNAs suitability for further molecular

8 244 analyses. PCR-based amplification of *stamp* gene identified the presence of ‘*Ca. P. solani*’ in 127

9 245 out of 400 individuals. Infection rate was >40% in *H. obsoletus*, *E. variegatus*, and *P. spumarius*,

10 246 >30% in *D. europaea*, *C. viridis* and *E. incisus*, and >10% in *P. alienus/confinis*. The phytoplasma

11 247 was not identified in the least abundant species *L. striatella*, *D. hamata* and *A. makarovi* (Table 1);

12 248 these latter three species were thus not included in microbiota analyses. For each of the other seven

13 249 insect species, the number of ‘*Ca. P. solani*’-infected and -non-infected specimens selected for

14 250 microbiota analyses is reported in Table 1.

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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

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3 274 distribution of the different insect species both infected and non-infected groups were characterized
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5 275 in terms of the relative taxonomic abundance. A total of 18 phyla, 46 classes, 58 orders, 89 families,
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7 276 100 genera and 35 species (of which a total of 277 with an unidentified taxa).
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10 278 **Core microbiome**

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12 279 In order to highlight the existence of an identifiable common core microbiome, the group of
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14 280 members shared among the microbial community of the infected and non-infected groups of the
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16 281 different insect species were identified. The bacterial communities in these insect populations are
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18 282 clearly distinct and do not share a common core as no single OTU is shared (i) among individuals
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20 283 of all insect species regardless of infection, (ii) among infected individuals regardless of insect
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22 284 species, (iii) among non-infected individuals regardless of insect species. Venn diagrams were used
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24 285 to represent the number of OTUs found exclusively in the infected group, non-infected group, or
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26 286 shared between the two groups (Fig. 2). For most of the analyzed species, a common trend can be
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28 287 identified with infected individuals showing a much higher number of unique OTUs compared to
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30 288 non-infected samples. This difference is particularly pronounced in *E. incisus* and *E. variegatus*
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32 289 (Fig. 2 and 3). This is true for all species, except *D. europaea* and *P. spumarius*, for which the
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34 290 number of unique OTUs in infected and non-infected samples is very similar (Fig. 2). Interestingly,
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36 291 regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs
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38 292 shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65
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40 293 shared OTUs (Fig. 2).

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42 294 Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between
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44 295 infected and non-infected in all species. Other relevant bacterial genera that are core between
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46 296 infected and non-infected in particular species are *Cronobacter* and *Sodalis* (*C. viridis*), *Erwinia* (*P.*
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48 297 *spumarius*), *Propionibacterium* (*E. variegatus*), *Purcellliella* (*H. obsoletus*), and *Rickettsia* (*H.*
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50 298 *obsoletus*).

51 300 **Bacterial community structure**

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53 301 Venn diagram representation showed a qualitative difference among OTUs identified in infected
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55 302 and non-infected individuals in the species, without considering the vital quantitative aspect in
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57 303 describing the community structure. To compare the microbial community structure among the ‘*Ca.*
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59 304 *P. solani*’ infected and non-infected individuals within and among insect species, principle
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61 305 coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis
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63 306 dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 4).
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3 307 The graph shows that the species is a major driver of diversity among the microbial communities, as
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5 308 each species tends to form a separate cluster. From this analysis, two groups of insects can be
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7 309 identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on
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9 310 species alone, with the single samples of infected and non-infected insects overlapping and mixing
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11 311 with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct
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13 312 clusters based on species for non-infected samples, but the infected samples do form clusters based
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15 313 on species, distinct from the non-infected samples within the same species. These results were
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17 314 confirmed by an Adonis multivariate analysis of variance, showing that there are statistically
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19 315 significant differences between the structure of the community in infected and non-infected samples
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21 316 of *E. incisus* ($p=0.001$), *E. variegatus* ($p=0.013$) and *P. alienus/confinis* ($p=0.006$), while no
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23 317 significant differences were found in the other four species.

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24 319 **Bacterial abundance and distribution**

25 320 The composition in taxa of the microbial communities according to the different insect species as
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27 321 well as the different infection status are reported in the bar plots in Fig. 5. All detected OTUs could
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29 322 be assigned to one of ten phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi,
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31 323 Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Proteobacteria, or Tenericutes (Fig.
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33 324 5A). In most analyzed samples, the most abundant phylum is Bacteroidetes, which can compose up
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35 325 to 99% of the total community, as for the non-infected *E. variegatus*. This dominance of
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37 326 Bacteroidetes is seen in all non-infected samples, except for *H. obsoletus*, and also in some infected
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39 327 insect species: *D. europaea*, *P. spumarius*, and *P. alienus/confinis*. The second most abundant
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41 328 phylum in most samples is Proteobacteria: this phylum is the most abundant in *H. obsoletus*, both
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43 329 infected and non-infected, and is also highly abundant also in *C. viridis*, *D. europaea*, and *P.*
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45 330 *spumarius*, both in infected and non-infected samples. While mostly absent in non-infected
46
47 331 samples, the phyla Actinobacteria and Firmicutes are found with higher abundance in the infected
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49 332 samples of *E. incisus*, and *E. variegatus*. Bacteria belonging to the phylum Cyanobacteria are found
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51 333 only in the infected samples of *E. incisus* and *E. variegatus*.

50 334 In most of the examined insect species, the microbial community was composed of
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52 335 members of few genera, but at very high abundance. In fact, as it can be seen by comparison of Fig.
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54 336 5A and 5B, almost the entire abundance of the Bacteroidetes phylum can be ascribed to the genus
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56 337 *Sulcia* alone. Likewise, the Tenericutes abundance is due uniquely to the presence of OTUs of ‘*Ca.*
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58 338 *Phytoplasma*’ and, in *H. obsoletus*, the abundance of Proteobacteria overlaps with the abundance of
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60 339 *Purcellliella*. In contrast, the microbiota of *E. incisus* shows many more genera, but the 12 most
60 340 abundant ones only cover 60% of the abundance, while the rest are less abundant genera. Regarding

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3 341 'Ca. Phytoplasma' OTUs, they are found exclusively in infected individuals of all species, but with
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5 342 high abundance (>1%) only in *E. incisus*, *E. variegatus*, and *P. alienus/confinis* (Table 3).

6 343 Comparing the infected and non-infected abundance of different genera, it emerges that for
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8 344 some species there are no changes, or very little changes, in the structure of the bacterial
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10 345 community in the presence of absence of 'Ca. Phytoplasma solani': *C. viridis*, *D. europaea*, and *P.*
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12 346 *spumarius* (Fig. 5B). For the main host, *H. obsoletus*, the community itself does not seem to
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14 347 undergo great variations in quality, with the addition of only *Rickettsia* in infected samples, but the
15 348 relative abundance of the members of the community are vastly different. Similarly, for *P.*
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17 349 *alienus/confinis* the community only shows the addition of 'Ca. Phytoplasma' between healthy and
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19 350 infected samples, but the abundance of OTUs belonging to this genus is very high, suggesting a
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21 351 strong interaction between this plant pathogen, the host, and the microbial community already
22 352 present in the host. For the remaining examined species (*E. incisus*, *E. variegatus*) the infection by
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24 353 the phytoplasma is accompanied by a radical change in the microbial community (Fig. 5B).

25 354 Regarding the bacterial genera that were of particular interest in this study, it can be seen
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27 355 that (i) the 'Ca. Phytoplasma'-antagonistic *Dyella* is not found in any of the examined insect
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29 356 species. (ii) *Wolbachia* is found in non-infected specimens of all vector species, but with high
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31 357 abundance (>1%) only in *D. europaea*, *E. incisus*, *H. obsoletus*, and *P. spumarius*; in all examined
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33 358 vector species, with the exception of *D. europaea*, the abundance of this genus is reduced in the
34 359 infected samples, to the degree of disappearing entirely from the community for *E. incisus* and *E.*
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36 360 *variegatus*. *Wolbachia* is not found in *C. viridis* regardless of phytoplasma infection (Table 3). (iii)
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38 361 The mutualistic symbiont *Sulcia* makes up for the majority of the microbiota in non-infected
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40 362 specimens of all insects, except *H. obsoletus*. Within phloem-feeders, it showed an abundance
41 363 >95% in *E. incisus*, *E. variegatus* and *P. alienus/confinis*, ~75% in *D. europaea*, and ~30% in *H.*
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43 364 *obsoletus*. Within xylem-feeders (*C. viridis* and *P. spumarius*), it showed an abundance <75%. With
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45 365 the exception of the xylem-feeders and *D. europaea*, its abundance is greatly reduced in infected
46 366 samples, compared to non-infected samples of the same species (Table 3).
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49 368 **DISCUSSION**

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51 369 The insect survey and molecular identification of 'Ca. P. solani', conducted in this study, confirmed
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53 370 the presence of abundant populations and the unusually high infection rate (>10%) in 2018 for the
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55 371 main vector *H. obsoletus* and for a majority of the insect species recently reported as vectors
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57 372 (Quaglino *et al.* 2019). If the scenario of containing Bois noir disease in vineyards was already
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59 373 bleak due to the high polyphagia of the established insect vectors, the addition of several more
60 374 vectors is leading to the idea that there are no options to implement any traditional containment

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3 375 strategy against this disease, its pathogen, or vectors. A comprehensive and thorough investigation
4 of the bacterial diversity in ‘*Ca. P. solani*’ insect vectors is essential for understanding how this
5 376 pathogen interacts with its hosts and their microbiota, possibly leading to the development of
6 377 effective prevention and treatment strategies based on the management of the bacterial community
7 378 in the vectors.
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12 380 This study analyzes the bacterial community present in insects associated to ‘*Ca. P. solani*’
13 381 collected in vineyards in northern Italy, including the main vector *H. obsoletus*, five newly reported
14 382 vector species (*D. europaea*, *E. incisus*, *E. variegatus*, *P. spumarius*, and *P. alienus/confinis*) and a
15 383 species that is known to host the phytoplasma but not to transmit it, *C. viridis*. In addition to
16 384 investigating and describing the bacterial community found in these insects, both when they’re
17 385 infected with ‘*Ca. P. solani*’ and when they aren’t, the study focuses on the presence of specific
18 386 genera of bacteria that have been reported as potentially essential for the survival of the insect
19 387 (genus *Sulcia*), as potential parasites of the vectors (genus *Wolbachia*), or as antagonistic towards
20 388 the phytoplasma (genus *Wolbachia* and *Dyella*).

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29 389 In comparison with previous studies on the topic of the bacterial communities associated to
30 390 insect vectors of ‘*Ca. P. solani*’, this study uses a more modern technique than those previously
31 391 employed [LH-PCR, DGGE (Gonella *et al.* 2011); sequencing with Roche 454 (Iasur-Kruh *et al.*
32 392 2017)] and extends the range of investigation to more vectors, instead of analyzing just *H.*
33 393 *obsoletus*.

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36 394 Starting from the parameters of alpha-diversity, it is found that in these insects the microbial
37 395 communities do not have a high diversity, showing a low number of different OTUs that dominate
38 396 the whole community. This is particularly true for the non-infected samples that showed less than
39 397 20 different OTUs for most of the analyzed species. This result is in accordance with what was
40 398 previously presented regarding the bacterial communities of phloem-/xylem-feeding insects, and it
41 399 is hypothesized that this is due to their extremely specialized diet which (i) requires specific
42 400 metabolic processes to implement the insect’s own and ensure survival and (ii) comes from a
43 401 compartment of the plant that is colonized only by very specialized bacteria and therefore acts as a
44 402 low-diversity reservoir from which the insects ingest bacteria (Colman *et al.* 2012; Jing *et al.* 2014;
45 403 Overholt *et al.* 2015).

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55 404 For most species there is no difference in the alpha-diversity parameters between ‘*Ca. P.*
56 405 *solani*’ infected and non-infected specimens, indicating that the presence of the pathogen does not
57 406 lead to a major change in the qualitative composition of the community. Still, for *E. incisus* and *E.*
58 407 *variegatus* a statistically significant increase was observed for all parameters in the infected
59 408 specimens, compared to the non-infected.

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3 409 The analysis of abundance of the different taxa in the insect species in general revealed
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5 410 microbial communities with low diversity, in which only a handful of genera were present with
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7 411 high abundance: *Bacillus* (Firmicutes), ‘*Candidatus Phytoplasma*’ (Tenericutes), *Cronobacter*
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9 412 (*Proteobacteria*), *Erwinia* (*Proteobacteria*), *Propionibacterium* (*Actinobacteria*), *Purcellliella*
10 413 (*Proteobacteria*), *Rickettsia* (*Proteobacteria*), *Sodalis* (*Proteobacteria*), *Staphylococcus* (*Firmicutes*),
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12 414 *Sulcia* (*Bacteroidetes*), and *Wolbachia* (*Proteobacteria*).

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14 415 The results obtained on the description of the bacterial microbiota of *E. incisus* and *P.*
15 416 *alienus/confinis* agree with what was previously reported by Kobińska *et al.* (2018), who indicated a
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17 417 microbial community dominated by *Sulcia* for these species.

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19 418 Regarding *H. obsoletus*, our results that highlighted the presence of the genera *Sulcia*,
20 419 *Wolbachia*, and *Purcellliella* confirming data previously obtained by Bressan *et al.* (2009) and
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22 420 *Gonella et al.* (2011) in northern Italy, but not those obtained by Iasur-Kruh *et al.* (2017) in Israel.
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24 421 This latter study determined, using both classical and molecular microbiology methods, that the
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26 422 genus *Sulcia* was the most abundant in *H. obsoletus*, followed by *Pectobacterium*. These
27 423 differences may be explained by several variables, such as the different techniques used, and the
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29 424 different geographical areas from which specimens were sampled, which leads to different climatic
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31 425 conditions and insect diet.

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33 426 The results obtained on *C. viridis*, with a high abundance of the genera *Sulcia* and *Sodalis*
34 427 are in accordance to those previously reported by Michalik *et al.* (2014). Intriguingly, these results
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36 428 also revealed that *C. viridis* has a unique microbiota compared to the other insect species analyzed:
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38 429 it has a more diverse composition, in which five different genera have a relevant level of abundance
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40 430 and it's the only species in which we find a high abundance of the genera *Cronobacter* and *Sodalis*.
41 431 These results suggest that either the higher diversity, leading to a more resilient bacterial
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43 432 community, or these specific genera of bacteria could play a role in determining the non-vector
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45 433 status of this insect. Further studies will be conducted to determine if these elements can indeed be
46 434 important and relevant for the development of an MRM strategy to reduce the spread of Bois noir.
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48 435 The results regarding the beta-diversity in each analyzed insect species, infected and non-
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50 436 infected, highlighted the presence of two different groups among the insect species: (i) insects for
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52 437 which the presence or absence of the phytoplasma did not cause a major restructuring of the
53 438 bacterial community, including the species *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius*;
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55 439 and (ii) insects for which the presence of the phytoplasma, not related to its abundance, caused a
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57 440 major change in the bacterial community, including the species *E. incisus*, *E. variegatus*, and *P.*
58 441 *alienus/confinis*. Interestingly, among the analyzed species, these three are the only ones belonging
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60 442 to the subfamily Deltoccephalinae. The microbiota associated with members of this subfamily is

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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

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3 476 reported that the presence of *Dyella*-like bacteria increases in the late stage of the season (Iasur-
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5 477 Kruh *et al.* 2017).

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7 478 In terms of abundance, the genus *Sulcia* was found to be the most abundant in all non-
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9 479 infected insect species except *H. obsoletus* where it was the second most abundant after
10 480 *Purcellliella*. This result is in agreement with Moran *et al.* (2005) who showed that several
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12 481 Auchenorrhyncha insect lineages, including Cicadomorpha and Fulgomorpha, house a single
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14 482 phylotype bacterium called ‘*Candidatus Sulcia muelleri*’. In the infected groups there was a
15 483 dramatic decrease in the genus *Sulcia*; except in the case of *C. viridis*, *D. europaea*, and *P.*
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17 484 *spumarius* where the reduction was quite low. This reduction in the abundance of *Sulcia* has several
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19 485 possible explanations: the first is that the interaction between the phytoplasma and other members
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21 486 of the microbiota lead to a rise of secondary mutualists, disadvantaging the primary mutualists such
22 487 as *Sulcia* (Heddi *et al.* 1998); a second hypothesis is related to the host’s immune response: it was
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24 488 demonstrated by Galetto *et al.* (2018) that the insect, *E. variegatus* in that study, can activate a
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26 489 strong immune response when interacting with a phytoplasma that is not the one that it usually
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28 490 transmits. This immune reaction could change the bacterial community inside the host drastically,
29 491 favoring more resistant bacteria, in particular Gram-positive species, as is seen in our study for *E.*
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31 492 *incisus* and *E. variegatus*. A third hypothesis is based on results obtained of *D. citri* and
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33 493 ‘*Candidatus Liberibacter asiaticus*’ by Vyas *et al.* (2015): this study demonstrated that the
34 494 phytopathogen could modulate free amino acids availability by interfering with hexamerin storage
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36 495 pathways by regulating expression of amino acid storage protein genes. Such evidence suggests that
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38 496 the reason why there is a dramatic reduction in genus *Sulcia*, which is heavily committed to amino
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40 497 acid production and encodes enzymes for synthesis of all amino acids required as animal nutrients,
41 498 is simply due to the fact that an infected insect does not need such a high abundance of this bacterial
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43 499 genus. On the other hand, sap-feeding insects rely heavily on the contribution of their obligate
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45 500 symbionts to maintain their metabolism (McCutcheon and Moran 2007). For this reason, the loss of
46 501 dominance by the beneficial *Sulcia* endosymbionts could instead prove to be detrimental to the
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48 502 insect’s fitness. More data on the fitness of the infected and non-infected insects would be needed to
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50 503 give a correct interpretation of this result. Genus *Wolbachia* tended to be present only in the non-
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52 504 infected specimens and was largely reduced in the infected insect species, except in the case of *D.*
53 505 *europaea*, in which the abundance of *Wolbachia* was higher in the ‘*Ca. P. solani*’ infected group.
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55 506 From these results, it becomes evident that the interaction is not just between the phytoplasma and
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57 507 *Wolbachia*, but that the insect species and the rest of the microbiota play a role in determining its
58 508 outcome. Still, considering that co-presence of phytoplasma and *Wolbachia* was not observed in the
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60 509 majority of the insect species, in general it is reasonable to conclude that a negative interaction

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CONCLUSION

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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.

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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

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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*.

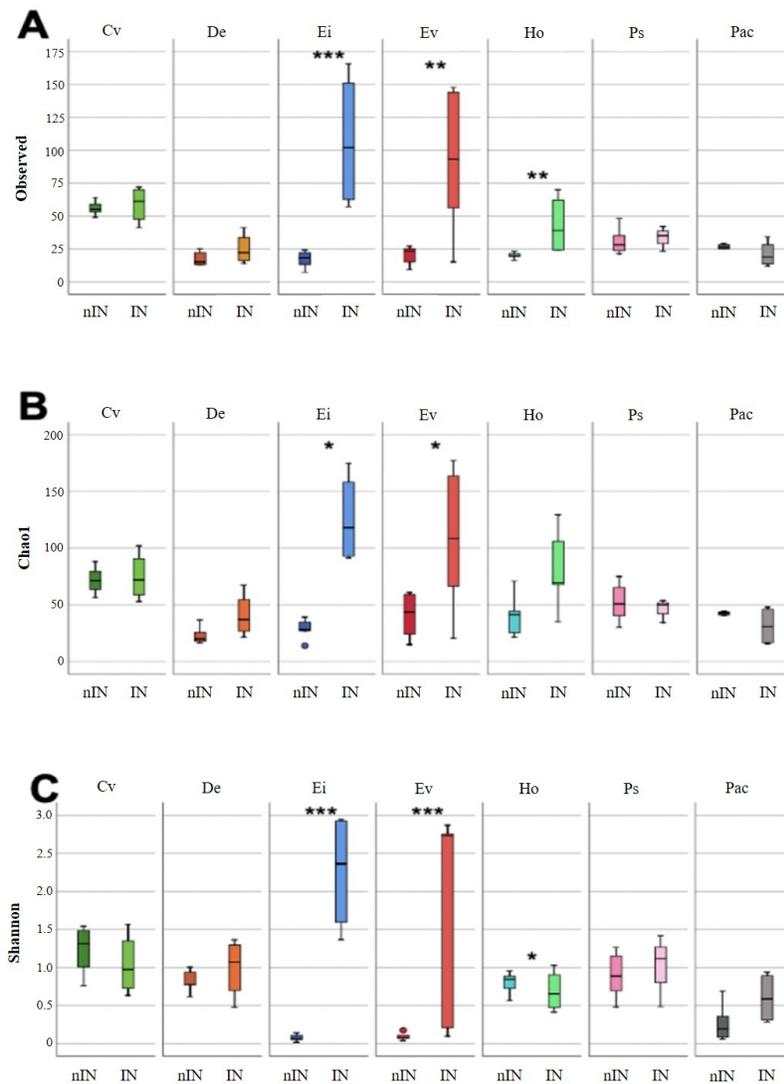


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182x260mm (150 x 150 DPI)

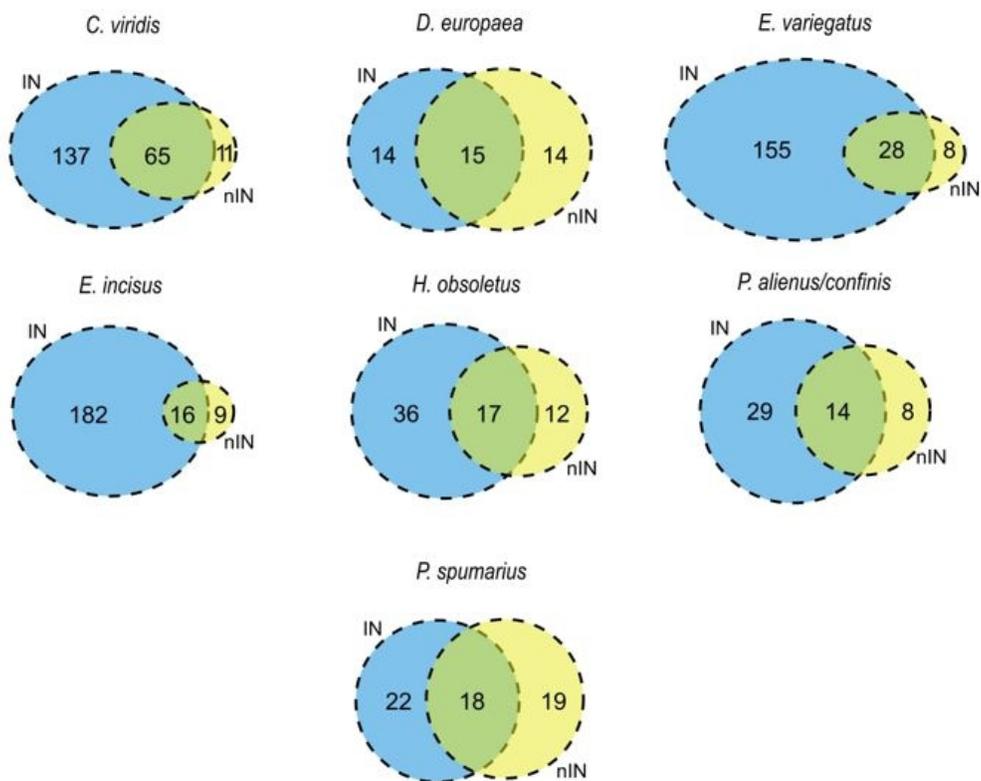


Figure 2. Venn diagrams showing the comparative distribution of OTUs in the different 'Ca. *P. solani*' infected and non-infected individuals within insect species. nIN: non-infected; IN: infected.

178x143mm (96 x 96 DPI)

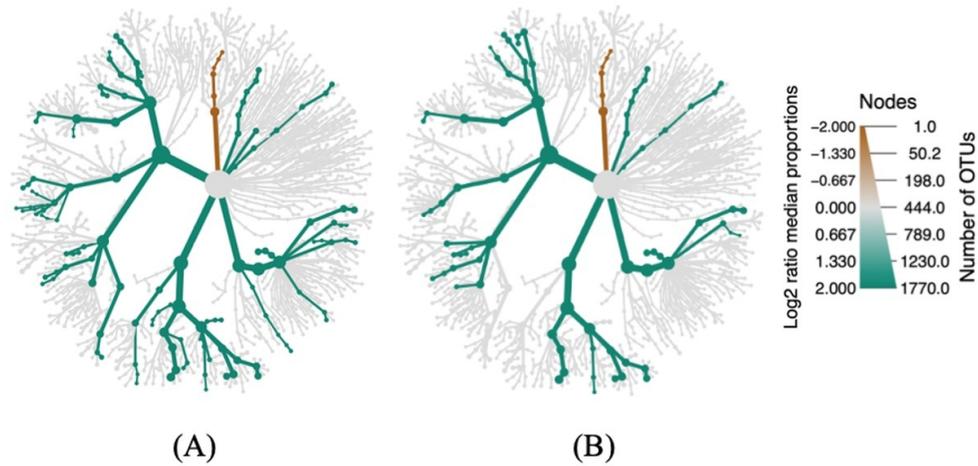


Figure 3. Differential heat tree showing differences in bacterial composition to the species level. The comparisons were made among the '*Ca. P. solani*' infected and non-infected groups. A, *E. incisus* where the green color represents the microbial community of the infected group and the brown color 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.

324x159mm (96 x 96 DPI)

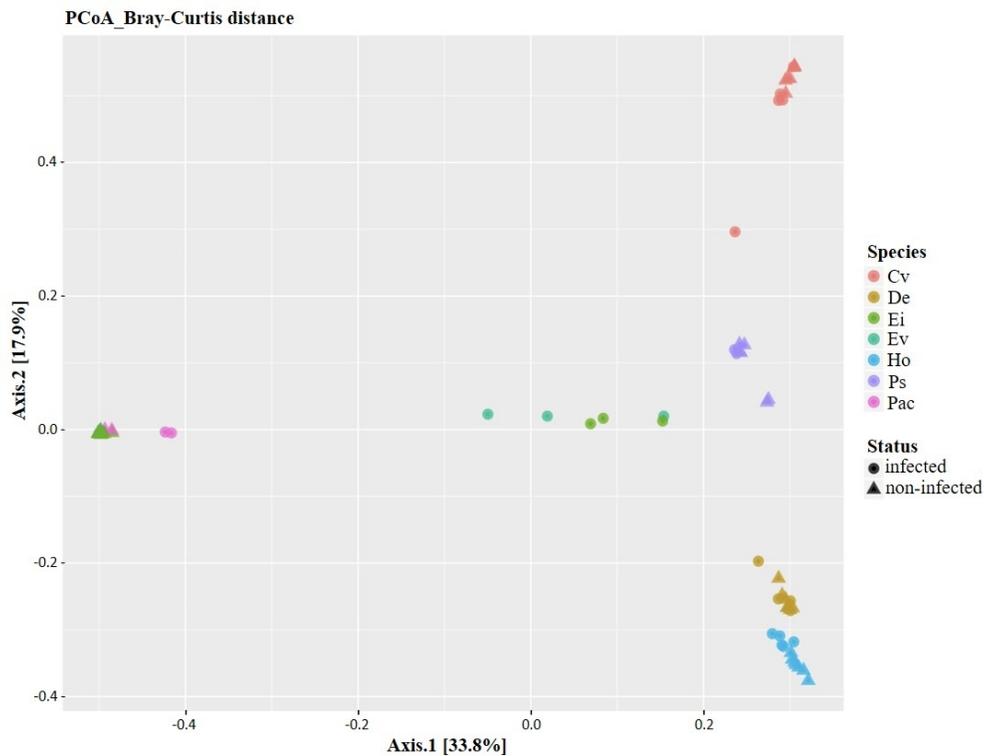


Figure 4. Beta-diversity. Graphs reporting the distribution of the samples according to beta-diversity calculated with a Bray-Curtis distance index. Different shape of the markers indicates different 'Ca. P. solani' infection status, different colors indicate different insect species, as indicated in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

183x141mm (150 x 150 DPI)

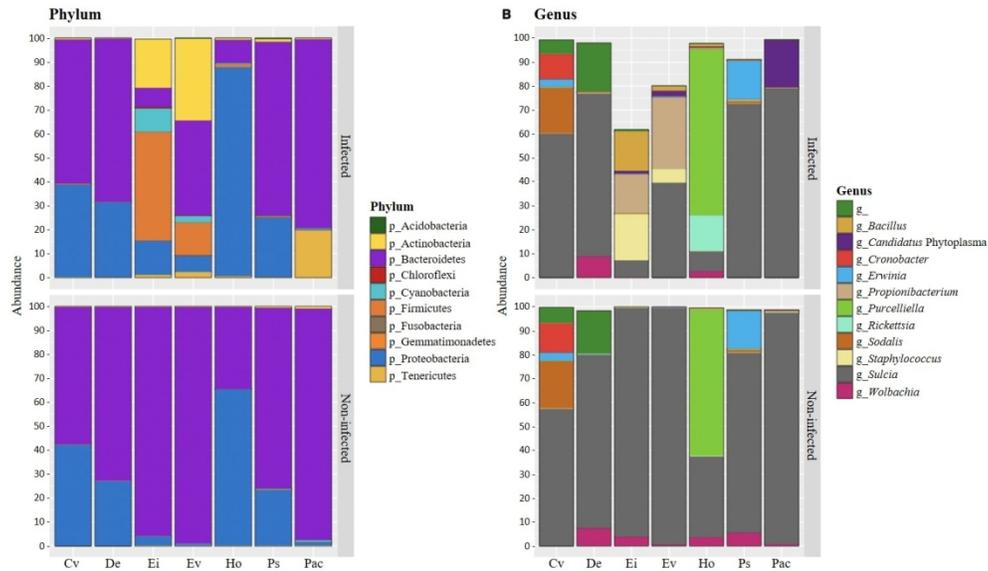


Figure 5. Relative abundance of operational taxonomic units at different levels: (A) phylum, (B) genus. Cv: *Cicadella viridis*; De: *Dictyophara europaea*; Ei: *Euscelis incisus*; Ev: *Euscelidius variegatus*; Ho: *Hyalesthes obsoletus*; Ps: *Phyllaenus spumarius*; Pac: *Psammotettix alienus/confinis*.

267x156mm (150 x 150 DPI)

1 Bacterial microbiota associated with insect vectors of grapevine Bois noir disease in 2 relation to phytoplasma infection

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4 **Abdelhameed Moussa^{1,2,#}, Alessandro Passera^{1,#}, Francesco Sanna³, Monica Faccincani⁴,
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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)
<i>Aphrodes makarovi</i>	6	0	0	0	0
<i>Cicadella viridis</i>	39	13	33	8/6	8/5
<i>Dicranotropis hamata</i>	8	0	0	0	0
<i>Dictyophara europaea</i>	40	15	38	9/5	9/4
<i>Euscelidius variegatus</i>	75	32	43	7/7	7/5
<i>Euscelis incisus</i>	59	18	31	9/5	9/4
<i>Hyalesthes obsoletus</i>	47	21	45	7/7	7/5
<i>Laodelphax striatella</i>	16	0	0	0	0
<i>Philaenus spumarius</i>	39	16	41	7/7	7/3
<i>Psammotettix alienus/confinis</i>	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
<i>C. viridis</i>	Infected	5	96729	202
	non-infected	8	143399	76
<i>D. europaea</i>	Infected	4	12803	29
	non-infected	9	25528	29
<i>E. incisus</i>	Infected	4	37284	198
	non-infected	9	33499	27
<i>E. variegatus</i>	Infected	5	34157	183
	non-infected	7	28210	36
<i>H. obsoletus</i>	Infected	5	30474	53
	non-infected	7	36010	29
<i>P. spumarius</i>	Infected	3	5609	40
	non-infected	7	12946	37
<i>P. alienus/confinis</i>	Infected	4	11134	43
	non-infected	6	19684	22

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

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

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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 illegible. 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

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3 number of different OTUs in infected and non-infected insect individuals do not
4 correspond to a change in microbiota structure (beta diversity analysis, renumbered
5 Figure 4) because the majority of OTUs included in Venn diagram are poorly
6 represented within the microbiota. Furthermore, some of these “unique” OTUs in Venn
7 diagram belong to the same phylum/genera, and therefore they are included in the same
8 group of abundance analysis (renumbered Figure 5).
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11 For clarity, in order to eliminate this apparent contradiction, we changed the order of
12 presentation of the results. We switched the order of the paragraphs to present first all
13 the data related to descriptive analysis (alpha diversity, Venn diagrams), and then the
14 quantitative ones (beta diversity, abundance). This flow reasoning was explained in the
15 Results (lines 301-306) as follows: “Venn diagram representation showed a qualitative
16 difference among OTUs identified in infected and non-infected individuals in the
17 species, without considering the vital quantitative aspect in describing the community
18 structure. To compare the microbial community structure among the ‘*Ca. P. solani*’
19 infected and non-infected individuals within and among insect species, principle
20 coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-
21 Curtis dissimilarity, which considers the abundance of shared and unique OTUs (Fig.
22 4)”.
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28 **Comment 6.** Figure 6 legend. Again, there is not enough information provided. The
29 labels IN and nIN must be explained. Moreover, this figure does not show infected
30 versus non-infected species as is currently stated, but infected vs. non-infected
31 individuals within a species.
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33 **Answer 6.** We modified the legend of Figure 6, renumbered as Figure 2, as suggested
34 (File “Figure Legends”, lines 24-25).
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37 **Comment 7.** Line 225: Please show the data.

38 **Answer 7.** We deleted “data not shown” because it was wrong. The results were already
39 included in the text as follows: “Bacterial 16S rDNA fragment 27F/1492R was amplified
40 by the TNAs extracted from all insect specimens and not in the negative control,
41 evidencing the TNAs suitability for further molecular analyses” (lines 243-245).
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45 **Comment 8.** Lines 366+367: It is unclear what these several considerations are. The text
46 simply goes on afterwards. If you want to highlight key considerations then it would
47 probably make sense to number them. e.g. First, alpha diversity... Second, ...
48

49 **Answer 8.** Reading the manuscript carefully, we noticed that this sentence was
50 unnecessary and inappropriate. Thus, we deleted it in the revised text.
51

52 **Comment 9** (minor comments).

- 53 - Line 25: Remove “therefore” because there is no logic link to the preceding sentence.
- 54 - Line 45: Change to “depend for food”.
- 55 - Line 221: This reads odd, as you cannot study species, which have not been captured.
56 Please revise.
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58 **Answer 9.** We fixed the text in accordance with these comments.
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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

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

10 This work should be cited in the corresponding section.

11 **Answer 12.** We thank the reviewer for these insights and suggestions on *Wolbachia*.

12 Considering these points, we expanded the sections regarding *Wolbachia* and its role, in
13 particular the abstract (lines 33-35), introduction (lines 107-119), the results (lines 356-
14 360), discussion (line 388; lines 503-513), and conclusions (lines 530-534).

15 Concerning the study by Krstic and colleagues, suggested by the reviewer, we
16 considered their results and hypotheses in the role of *Wolbachia* in *D. europaea* as
17 vector of flavescence dorée phytoplasma, and cited this work accordingly. However, we
18 did not directly compare our results with those by Krstic because our study focused on a
19 quantitative bacterial microbiota analysis conducted on insects sampled in a single
20 location, while those reported by Krstic are qualitative analyses carried out on insects
21 sampled in various locations in different countries, with a main focus on the
22 epidemiological significance of the presence of *Wolbachia* in different populations.
23 These differences in methodology, aim, and scope made it impossible, in our opinion, to
24 compare the results of the two studies. Also, our results are not in disagreement with
25 those by Krstic as also in that study (supplementary tables) some individuals infected
26 both by *Wolbachia* and phytoplasma are reported.

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

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

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5 **Comment 22.** Line 334- on what figure the description is based on? For example- from
6 fig 3B, Propionibacterium is not shared by Ev infected and not infected, same is for
7 Rickettsia in HO. please correct the description.

8 **Answer 22.** The statement is based on the OTUs included in the Venn diagram
9 (renumbered Figure 2), and it is a description of the OTUs that make up the number of
10 shared OTUs between infected and non-infected individuals in the same species. To
11 avoid confusion between this descriptive analysis and the quantitative analysis in the
12 interpretation of the text, the order of the results has been changed, presenting the Venn
13 diagrams and the description of shared OTUs before the quantitative results.
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17 **Comment 23.** Fig 1. Line 248- “The Shannon index, indicating the evenness of species
18 distribution ranges from a minimum of 0.089 in non-infected *E. variegatus*, to a
19 maximum of 2.25 in infected *E. incisus*.” Explain how these indices were calculated
20 regarding the scale in fig 1C.

21 Add the statistical meaning of * or ***.

22 Also- 65 and 82 are posted in the fig. near EI and EV non-infected Shanon and Chao-1
23 columns. What is the meaning of these numbers?

24 **Answer 23.** We fixed the scale in y-axis of Figure 1C. We deleted the numbers 65 and
25 82 from the Figure (they were inserted for a mistake). The legend of Figure 1 was
26 modified indicating the species acronyms and the statistical meaning of *, **, *** (File
27 “Figure Legends”, lines 18-22).
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33 **Comment 24.** Fig 2 –the font of species names is too small to read.

34 **Answer 24.** In accordance with the comments raised by the Editor, we preferred to
35 delete this figure and maintain the Figure 4, renumbered as Figure 3 in the revised
36 manuscript, including two trees comparing the bacterial microbiota associated with
37 infected vs non-infected individuals of the insects *E. incisus* (A) and *E. variegatus* (B).
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41 **Comment 25.** Line 432 -from fig 3B, there is dramatic decrease in *Sulcia* in Ho, Ev, Ei.
42 In the other 3 species (Cv, De, Ps), there is no big difference in abundance of *Sulcia*
43 between infected and non infected. Also the change in Pac is rather small. However the
44 number of phytoplasma OTU’s in Pac were the highest. Please refer to this point.
45 Also, phytoplasma OTU were not observed in Ho, the authors have to explain their
46 conclusion that the reduction abundance of in *Sulcia* and *Wolbachia* is related to
47 phytoplasma presence.
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50 **Answer 25.** To clarify these points, we added a new table in the text (Table 3), showing
51 the relative abundance of bacterial genera relevant for our study (‘*Ca. Phytoplasma*’,
52 *Sulcia*, *Wolbachia*), showing that there is correlation between phytoplasma presence and
53 decrease of *Sulcia* and *Wolbachia* (Results, lines 354-366; Discussion, line 439).
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56 **Comment 26.** The main concern is that phytoplasma was shown by PCR in 7/10 species
57 but only in 3/10 by OTUs. Also phytoplasma was not detected by OTU in Ho which is
58 the main vector of BN and showed the highest rate of infection by PCR. Please refer to
59 this point.
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3 **Answer 26.** Please, see answer to Comment 19.
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6 **Comment 27.** The author need to explain why it is concluded that a reduction in *Sulcia*
7 in infected insects is a marker for increased efficiency in metabolite utilization of the
8 insect. From the same evidence it can be explained that the fitness is reduced in the
9 presence of phytoplasma because the abundance of *Sulcia* in reduced.
10

11 **Answer 27.** We agree with the reviewers, the conclusion on the role of the reduction of
12 *Sulcia* was indeed too speculative. We mitigated the sentences regarding this
13 phenomenon throughout the text (lines 33-35; lines 495-502), and stated that further data
14 regarding the fitness of the insects should be obtained before a definitive interpretation
15 of this result can be given (lines 530-534).
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18 **Comment 28.** line 471- this is an hypothesis that has to be proved, not a conclusion
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20 **Answer 28.** Considering the points raised by the reviewers, we expanded the sections
21 regarding *Wolbachia* and its role, including the conclusions (lines 530-534).
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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'.

†Fabio Quaglino, <https://orcid.org/0000-0001-8866-0633>

ABSTRACT

Bois noir is ~~the prevalent~~ the prevalent ~~grapevine~~ grapevine disease ~~causing severe yield loss in vineyards worldwide.~~ 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,~~ ~~†~~ 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~~Auchenorrhyncha~~, 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,

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

74 The epidemiological cycle associated to Bois noir is extremely complex and was recently
75 discovered to include not only the most well-known vectors *Hyalesthes obsoletus* (Maixner 1994)
76 and *Reptalus panzeri* (Cvrkovic *et al.* 2014), but also other eight species: *Aphrodes makarovi*,
77 *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, *Euscelidius variegatus*,
78 *Laodelphax striatella*, *Phyllaenus spumarius*, and *Psammotettix alienus/confinis* (Quaglino *et al.*
79 2019).

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

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

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

100 A negative prospect for this strategy is that, as the interactions between environment, host,
101 and microbiota are very complex and influenced by several variables (Trivedi *et al.* 2015); Douglas
102 2015; Fonseca-García *et al.* 2016), more studies need to be conducted in the description of the
103 bacterial community associated to these vectors before its manipulation can become a viable option.
104 The positive prospect is that, since these phloem-feeding insects rely heavily on obligate bacterial

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3 105 symbionts to provide nutrients which are lacking in their unbalanced diet (Buchner 1965; Baumann
4 106 2005; Bourtzis and Miller 2006; Skidmore and Hansen 2017), ~~it is that hypothesized that~~ these
5 107 insects will be particularly susceptible to unbalances in their microbial community.

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8 108 A main actor in these obligate mutualistic interactions is ‘*Candidatus Sulcia muelleri*’, a
9 109 bacterial species that greatly reduced its genome as it evolved as an obligate symbiont; ~~;~~ moreover, it
10 110 and is documented to be strictly associated to leafhoppers and planthoppers, among other hosts
11 111 (Moran *et al.* 2005; McCutcheon *et al.* 2009). This bacterial species is involved in the synthesis of
12 112 several amino acids necessary for the insect host, ~~including histidine and methionine~~ (McCutcheon
13 113 and Moran 2007). Other mutualistic bacteria involved in these interactions belong to the genera
14 114 *Nasuia* and *Sodalis* (Kobińska *et al.* 2018).

15 115 Another bacterial genus interesting for MRM approach is ~~On the other side of the spectrum,~~
16 116 ~~we can find parasitic or antagonistic bacteria, such as those belonging to the genera *Wolbachia* and~~
17 117 ~~*Dyella*. *Wolbachia* is a genus of~~ ubiquitous bacterial endosymbionts associated with over 60% of
18 118 known insect species, as well as other arthropods and nematodes (Hosokawa *et al.* 2010; Zug and
19 119 Hammerstein 2012; Newton and Rice 2020). *Wolbachia* species are cytoplasmically inherited and
20 120 known as reproductive parasites due to their ability to manipulate reproduction such as sperm-egg
21 121 incompatibility (cytoplasmic incompatibility), parthenogenesis induction, male killing, and
22 122 feminization, making it a possible biocontrol agent against the vectors (Werren 1997; Stouthamer *et*
23 123 *al.* 1999; Werren *et al.* 2008; Brelsfoard and Dobson 2009; Chuche *et al.* 2017). Nevertheless,
24 124 several studies showed that *Wolbachia* can act as mutualistic towards insect hosts, modulating
25 125 nutrition and immune responses (Hosokawa *et al.* 2010; Iturbe-Ormaetxe *et al.* 2011; Newton and
26 126 Rice 2020). Moreover, recent studies proposed that *Wolbachia* can act as biocontrol agent of insect-
27 127 transmitted pathogens, including phytoplasmas, by increasing latency period and blocking pathogen
28 128 transmission is an interesting and promising biological control agent that can be used to stop or
29 129 prevent the transmission of several pathogens (Shaw *et al.* 2016; Chuche *et al.* 2017).

30 130 *Dyella*-like bacterium (DLB), gram-negative, aerobic, rod-shaped endophytic bacteria
31 131 belonging to the family Rhodanobacteraceae, can be acquired by feeding and has shown a potential
32 132 biocontrol activity against phytoplasmas and their cultivable relative *Spiroplasma melliferum*
33 133 (Iasur-Kruh *et al.* 2017, 2018). The possible mechanisms of DLB antagonism towards
34 134 phytoplasmas have been hypothesized to be (i) competition for nutrients or colonization niches, (ii)
35 135 induction of plant systemic resistance, (iii) secretion of plant growth hormones, or (iv) secretion of
36 136 phytoplasma growth inhibitory substances (Eljounaidi *et al.* 2016).

37 137 In this scenario, the current study aims to characterize through an Next Generation
38 138 Sequencing (NGS) approach the microbial-bacterial community associated with selected recently

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2
3 139 ~~identified ‘Ca. P. solani’ insect hosts, vectors (*A. makarovi*, *D. hamata*, *D. europaea*, *E. incisus*, *E.*~~
4 ~~*variegatus*, *L. striatella*, *P. spumarius*, and *P. alienus/confinis*), the main vector *H. obsoletus*, and~~
5 140 ~~one insect host but non-vector (*Cicadella viridis*), both infected and non-infected by the~~
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7 141
8
9 142 phytoplasma, with the following goals: (i) describe the ~~microbial-bacterial~~ communities in different
10 143 insect hosts of ‘Ca. P. solani’; (ii) determine whether the presence of ‘Ca. P. solani’ affects the
11
12 144 ~~microbial-bacterial~~ community, in particular if it can cause a dysbiosis (also called dysbacteriosis)
13
14 145 or increase diversity; (iii) evaluate the presence of possible antagonists towards the insect (e.g.
15 146 *Wolbachia* spp.) or phytoplasma (e.g. *Wolbachia* spp. and *Dyella*-like bacteria); (iv) investigate the
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17 147 effect of ‘Ca. P. solani’ on the obligate endosymbiont ‘Ca. Sulcia’ spp. The selected insects are
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19 148 the main vector *H. obsoletus*, newly reported vectors (phloem-feeders: *A. makarovi*, *D. hamata*, *D.*
20 149 *europaea*, *E. incisus*, *E. variegatus*, *L. striatella*, and *P. alienus/confinis*; xylem-feeder: *P.*
21 149 *spumarius*), and *Cicadella viridis*, one of the most abundant insects living in Italian vineyard,
22 150 harboring with high infection rate but not vectoring ‘Ca. P. solani’ (Quaglino *et al.* 2019), and
23
24 151 characterized by xylem-feeding activity. *C. viridis* was included in the study for comparing the
25
26 152 microbiota associated with xylem- and phloem-feeders, and investigating the phytoplasma influence
27 153 on the microbiota structure in comparison with vectors. *R. panzeri* was not among the selected
28
29 154 vectors because it is not found in the studied area.
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31 155

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33 156 Achieving the previously mentioned aims regarding the description of the bacterial
34 157 communities may help in devising MRM-based approaches to achieve the main objective of
35
36 158 biological control of ‘Ca. P. solani’ and its insect vectors.
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38 159

39 160 MATERIALS AND METHODS

43 162 Insect collection

44 163 Specimens of the insect species *A. makarovi*, *C. viridis*, *D. hamata*, *D. europaea*, *E. incisus*, *E.*
45 163 *variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius*, and *P. alienus/confinis* were captured by
46 164 sweep entomological net in mid-July 2018 in the Chardonnay organic vineyard (Franciacorta,
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48 165 Lombardy Region, North Italy; N 45°35'38.12", E 10°09'34.32") where new insect vectors of ‘Ca.
49
50 166 P. solani’ had previously been identified (Quaglino *et al.* 2019). Insect individuals were stored in
51 167 ethanol 90%, transferred to the lab for species identity confirmation by stereomicroscope based on
52
53 168 the taxonomic keys of den Bieman *et al.* (2011), and maintained in absolute ethanol at 4°C till use.
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55 169 Regarding the genus *Psammotettix*, given that the dichotomous keys are related only to males, the
56
57 170 species *P. alienus* and *P. confinis* were considered together.
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60 172

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

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3 207 undergo Illumina Mi Seq sequencing. These 96 samples were picked to ensure that at least five
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5 208 samples for each insect species were included in both the ‘*Ca. P. solani*’-infected and non-infected
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7 209 groups.

8 210 Next generation sequencing library preparations and Illumina Mi Seq sequencing were
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10 211 conducted by an external provider (Personal Genomics, Verona, Italy). The bacterial *16S rRNA*
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12 212 gene [hypervariable region V4](#) libraries ~~was~~ ~~were~~ prepared using the forward primer 515FB (5'-
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14 213 GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3'), and the
15 214 amplification of sequences belonging to mitochondria was blocked using a PNA blocker (Lundberg
16
17 215 *et al.* 2013). Metagenomic sequencing was performed using the Illumina Miseq 300PE sequencing
18
19 216 technology. Obtained reads were deposited in the EMBL-ENA under the project number
20
21 217 PRJEB38750.

22 218

23 24 219 **Processing of high-throughput sequencing data**

25 220 The raw sequencing reads were initially filtered, to remove low quality sequences, trim primers and
26
27 221 Illumina adapters. The initial quality control of the reads was performed with FastQC v0.11.5.
28
29 222 Primers were trimmed with the cutadapt tool version 1.14 (Martin 2011) while adapters were
30
31 223 trimmed with Sickle version 1.33 (<https://github.com/najoshi/sickle>) and Scythe version 0.991
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33 224 (<https://github.com/vsbuffalo/scythe>). The obtained reads were analyzed using the QIIME 2
34 225 pipeline (Bolyen *et al.* 2019) in order to assign them to OTUs. Allocation to OTUs and clustering
35
36 226 were performed using uclust with a minimum similarity of 97% (default). Identified OTUs from
37
38 227 representative sequences were aligned to Green-genes (<http://greengenes.lbl.gov/>) using R-studio.
39 228 Chloroplast and mitochondria (these constituted only 1-2% in some samples) were filtered as well
40
41 229 as rare OTUs (i.e., singletons and OTUs < 10). The resulting OTU table was then used for the
42
43 230 subsequent analyses.

44 231

45 46 232 **Diversity and statistical analysis**

47 233 After quality filtering and rarifying to 1600 sequences per sample, Alpha-diversity indices
48
49 234 (Shannon index, ChaoI and observed OTU) were calculated to ensure that enough sequencing
50
51 235 coverage had been achieved by using BiocManager package implemented in the R software (R
52
53 236 Project 3.0.2; <http://cran.rproject.org/>). Observed, Chao1 (Chao 1984) and Shannon H' index
54
55 237 (Shannon 1948) were considered for the aforementioned features. Alpha diversity indices were
56
57 238 compared between different insect species groups ('*Ca. P. solani*' infected or non-infected). Shapiro
58
59 239 test was performed for data normality followed by ANOVA in the case of Observed richness
60 240 whereas Kruskal test was used for Chao1 and Shannon H' index. Welch t-test was carried out to

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2
3 241 compare between the infected and non-infected groups of individual species. Beta diversity was
4
5 242 assessed by Bray-Curtis (Bray and Curtis 1957) distance matrices and visualized by principal
6
7 243 coordinate analysis (PCoA). The PERMANOVA statistical analysis was performed to determine
8
9 244 the significance of microbial community differences among the different insect species and
10 245 infection status with controlled 10^5 permutations. Taxonomic abundance data was calculated using
11
12 246 the percentage abundance of OTUs present in the core microbiota. Heat tree was used to plot all the
13
14 247 OTUs present in the dataset using the 'metacoder' package. Taxonomic data were plotted using heat
15 248 trees in which the size and color of tree parts correspond to reads for each taxon as the size of each
16
17 249 taxon. ~~It also plots the number of OTUs assigned to each taxon in the overall dataset as color.~~

20 251 RESULTS

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22 252
23
24 253 **Insects collected and 'Ca. P. solani' infection rate.** A total of 400 ~~insect~~ individuals were
25
26 254 captured, ~~belonging to all the insect species under study.~~ The most abundant species were *E.*
27 255 *variegatus* (75 individuals), *P. alienus/confinis* (71) and *E. incisus* (59), while *L. striatella* (16), *D.*
28 256 *hamata* (8) and *A. makarovi* (6) were scarcely present (Table 1). Bacterial 16S rDNA fragment
29 257 27F/1492R was amplified by the TNAs extracted from all insect specimens and not in the negative
30
31 258 control, evidencing the TNAs suitability for further molecular analyses ~~(data not shown)~~. PCR-
32
33 259 based amplification of *stamp* gene identified the presence of 'Ca. P. solani' in 127 out of 400
34
35 260 individuals. Infection rate was >40% in *H. obsoletus*, *E. variegatus*, and *P. spumarius*, >30% in *D.*
36 261 *europaea*, *C. viridis* and *E. incisus*, and >10% in *P. alienus/confinis*. The phytoplasma was not
37
38 262 identified in the least abundant species *L. striatella*, *D. hamata* and *A. makarovi* (Table 1); these
39
40 263 latter three species were thus not included in microbiota analyses. For each of the other seven insect
41
42 264 species, the number of 'Ca. P. solani'-infected and -non-infected specimens selected for microbiota
43
44 265 analyses is reported in Table 1.

46 266 47 48 267 Bacterial diversity analysis

49
50 268 Poor quality sequences were obtained in twelve out of 96 insect specimens that were excluded from
51
52 269 further analyses (Table 1). Sequencing of the ~~v4-v5~~V4 region of the *16S rRNA* gene on the 'Ca. P.
53 270 solani' infected and non-infected group produced, after filtering out organellar sequences and rare
54
55 271 OTUs, a total of 527466 sequences belonging to 363 different OTUs. Out of all the obtained
56
57 272 sequences, 228190 belong to 'Ca. P. solani' infected group and 299276 to the non-infected group.
58
59 273 Number of sequences and OTUs obtained from the 'Ca. P. solani' infected and non-infected group
60 274 are reported in Table 2.

1
2
3 275 The alpha diversity indices of Observed, Chao1 and Shannon were used for this study as
4
5 276 shown in Fig. 1. The observed OTUs were considered to show the absolute richness. The values of
6
7 277 this parameter range from a minimum average of 17, found in non-infected *E. incisus* and *D.*
8
9 278 *europaea*, to a maximum of 106, found in infected *E. incisus*. The corrected estimation of richness
10 279 made through the Chao1 index are very close to the value of Observed for most samples, indicating
11
12 280 that the sequencing has reached an adequate depth, having very few singletons and a low number of
13
14 281 estimated undetected OTUs. The Shannon index, indicating the evenness of species distribution
15 282 ranges from a minimum of 0.089 in non-infected *E. variegatus*, to a maximum of 2.25 in infected *E.*
16
17 283 *incisus*. For *E. incisus*, *E. variegatus* and *H. obsoletus*, the number of Observed OTU and the
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19 284 Shannon index are significantly different between infected and non-infected samples, indicating
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21 285 that the presence of the phytoplasma has a strong effect on the alpha-diversity of the bacterial
22 286 community in these species. For all other considered insect species, no statistically significant
23
24 287 difference was found between these values for infected and non-infected groups. The bacterial
25
26 288 distribution of the different insect species both infected and non-infected groups were characterized
27 289 in terms of the relative taxonomic abundance. A total of 18 phyla, 46 classes, 58 orders, 89 families,
28
29 290 100 genera and 35 species (of which a total of 277 with an unidentified taxa) (Fig. 2).

31 291 32 292 **Core microbiome**

33 293 In order to highlight the existence of an identifiable common core microbiome, the group of
34 294 members shared among the microbial community of the infected and non-infected groups of the
35
36 295 different insect species were identified. The bacterial communities in these insect populations are
37
38 296 clearly distinct and do not share a common core as no single OTU is shared (i) among individuals
39
40 297 of all insect species regardless of infection, (ii) among infected individuals regardless of insect
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42 298 species, (iii) among non-infected individuals regardless of insect species. Venn diagrams were used
43
44 299 to represent the number of OTUs found exclusively in the infected group, non-infected group, or
45
46 300 shared between the two groups (Fig. 2). For most of the analyzed species, a common trend can be
47
48 301 identified with infected individuals showing a much higher number of unique OTUs compared to
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50 302 non-infected samples. This difference is particularly pronounced in *E. incisus* and *E. variegatus*
51
52 303 (Fig. 2 and 3). This is true for all species, except *D. europaea* and *P. spumarius*, for which the
53 304 number of unique OTUs in infected and non-infected samples is very similar (Fig. 2). Interestingly,
54
55 305 regardless of the total amount of OTUs found in different species, there are 14-28 core OTUs
56
57 306 shared between infected and non-infected samples, with the exception of *C. viridis*, which shows 65
58 307 shared OTUs (Fig. 2).
59
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3 308 Among the shared OTUs, only bacteria belonging to the genus *Sulcia* is found to be shared between
4 infected and non-infected in all species. Other relevant bacterial genera that are core between
5 309 infected and non-infected in particular species are *Cronobacter* and *Sodalis* (*C. viridis*), *Erwinia* (*P.*
6 310 *spumarius*), *Propionibacterium* (*E. variegatus*), *Purcellliella* (*H. obsoletus*), and *Rickettsia* (*H.*
7 311 *obsoletus*).

13 314 **Bacterial community structure**

15 315 Venn diagram representation showed a qualitative difference among OTUs identified in infected
16 and non-infected individuals in the species, without considering the vital quantitative aspect in
17 316 describing the community structure. To compare the microbial community structure among the ‘*Ca.*
18 *P. solani*’ infected and non-infected individuals within and among insect species, principle
19 317 coordinate analysis (PCoA) of beta diversity analysis was performed based on Bray-Curtis
20 318 dissimilarity, which considers the abundance of shared and unique OTUs (Fig. 4).

25 321 The graph shows that the species is a major driver of diversity among the microbial communities, as
26 322 each species tends to form a separate cluster. From this analysis, two groups of insects can be
27 323 identified: (i) *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius* form clusters based on
28 324 species alone, with the single samples of infected and non-infected insects overlapping and mixing
29 325 with one another; (ii) *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, instead, do not form distinct
30 326 clusters based on species for non-infected samples, but the infected samples do form clusters based
31 327 on species, distinct from the non-infected samples within the same species. These results were
32 328 confirmed by an Adonis multivariate analysis of variance, showing that there are statistically
33 329 significant differences between the structure of the community in infected and non-infected samples
34 330 of *E. incisus* (p=0.001), *E. variegatus* (p=0.013) and *P. alienus/confinis* (p=0.006), while no
35 331 significant differences were found in the other four species.

46 333 **Bacterial abundance and distribution**

48 334 The composition in taxa of the microbial communities according to the different insect species as
49 335 well as the different infection status are reported in the bar plots in Fig. 53. All detected OTUs
50 336 could be assigned to one of ten phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi,
51 337 Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Proteobacteria, or Tenericutes (Fig.
52 338 53A). In most analyzed samples, the most abundant phylum is Bacteroidetes, which can compose
53 339 up to 99% of the total community, as for the non-infected *E. variegatus*. This dominance of
54 340 Bacteroidetes is seen in all non-infected samples, except for *H. obsoletus*, and also in some infected
55 341 insect species: *D. europaea*, *P. spumarius*, and *P. alienus/confinis*. The second most abundant

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3 342 phylum in most samples is Proteobacteria: this phylum is the most abundant in *H. obsoletus*, both
4 343 infected and non-infected, and is also highly abundant also in *C. viridis*, *D. europaea*, and *P.*
5 344 *spumarius*, both in infected and non-infected samples. While mostly absent in non-infected
6 345 samples, the phyla Actinobacteria and Firmicutes are found with higher abundance in the infected
7 346 samples of *E. incisus*, and *E. variegatus*. ~~As expected, the phylum Tenericutes, which includes ‘Ca.~~
8 347 ~~*P. solani*’, is found with high abundance only in the infected samples and, even then, only in three~~
9 348 ~~species: *E. incisus*, *E. variegatus*, and *P. alienus/confinis*.~~ Bacteria belonging to the phylum
10 349 Cyanobacteria are found only in the infected samples of *E. incisus* and *E. variegatus*.

11
12 350 In most of the examined insect species, the microbial community was composed of
13 351 members of few genera, but at very high abundance. ~~In fact, As~~ it can be seen by comparison of
14 352 Fig. ~~53A~~ and ~~53B~~, almost the entire abundance of the Bacteroidetes phylum can be ascribed to the
15 353 genus *Sulcia* alone. Likewise, the Tenericutes abundance is due uniquely to the presence of OTUs
16 354 of ‘*Ca. Phytoplasma*’ and, in *H. obsoletus*, the abundance of Proteobacteria overlaps with the
17 355 abundance of *Purcellliella*. ~~In contrast, In~~ the microbiota of *E. incisus* ~~there are shows~~ many more
18 356 genera, but the ~~twelve-12~~ most abundant ones only cover 60% of the abundance, while the rest are
19 357 less abundant genera. ~~Regarding ‘Ca. Phytoplasma’ OTUs, they are found exclusively in infected~~
20 358 ~~individuals of all species, but with high abundance (>1%) only in *E. incisus*, *E. variegatus*, and *P.*~~
21 359 ~~*alienus/confinis* (Table 3).~~

22 360 Comparing the infected and non-infected abundance of different genera, it emerges that for
23 361 some species there are no changes, or very little changes, in the structure of the bacterial
24 362 community in the presence of absence of ‘*Ca. Phytoplasma solani*’: *C. viridis*, *D. europaea*, and *P.*
25 363 *spumarius* (Fig. ~~53B~~). For the main host, *H. obsoletus*, the community itself does not seem to
26 364 undergo great variations in quality, with the addition of only *Rickettsia* in infected samples, but the
27 365 relative abundance of the members of the community are vastly different. Similarly, for *P.*
28 366 *alienus/confinis* the community only shows the addition of ‘*Ca. Phytoplasma*’ between healthy and
29 367 infected samples, but the abundance of OTUs belonging to this genus is very high, suggesting a
30 368 strong interaction between this plant pathogen, the host, and the microbial community already
31 369 present in the host. For the remaining examined species (*E. incisus*, *E. variegatus*) the infection by
32 370 the phytoplasma is accompanied by a radical change in the microbial community (Fig. ~~5B4~~).

33 371 Regarding the bacterial genera that were of particular interest in this study, it can be seen
34 372 that (i) the ‘*Ca. Phytoplasma*’-antagonistic *Dyella* is not found in any of the examined insect
35 373 species; (ii) ~~the possibly insect-antagonistic *Wolbachia*~~ is found ~~in non-infected specimens of all~~
36 374 ~~vector species, but with high abundance (>1%) only in *D. europaea*, *E. incisus*, *H. obsoletus*, and~~
37 375 *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 *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, wWith the exception of the xylem-feeders and of *C. viridis*, *D. 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. incisus* ($p=0.001$), *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*), *Purcellliella* (*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 vector 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

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3 443 (genus *Sulcia*), as potential parasites of the vectors (genus *Wolbachia*), or as antagonistic towards
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5 444 the phytoplasma (genus *Wolbachia and Dyella*).

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7 445 In comparison with previous studies on the topic of the bacterial communities associated to
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9 446 insect vectors of ‘*Ca. P. solani*’, this study uses a more modern technique than those previously
10 447 employed [LH-PCR, DGGE (Gonella *et al.* 2011); sequencing with Roche 454 (Iasur-Kruh *et al.*
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12 448 2017)] and extends the range of investigation to more vectors, instead of analyzing just *H.*
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14 449 *obsoletus*.

15 450 ~~The results obtained from the investigation of the bacterial microbiota of these insect species~~
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17 451 ~~allows the formulation of several considerations.~~

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19 452 Starting from the parameters of alpha-diversity, it is found that in these insects the microbial
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21 453 communities do not have a high diversity, showing a low number of different OTUs that dominate
22 454 the whole community. This is particularly true for the non-infected samples that showed less than
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24 455 20 different OTUs for most of the analyzed species. This result is in accordance with what was
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26 456 previously presented regarding the bacterial communities of phloem-/xylem-feeding insects, and it
27 457 is hypothesized that this is due to their extremely specialized diet which (i) requires specific
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29 458 metabolic processes to implement the insect’s own and ensure survival and (ii) comes from a
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31 459 compartment of the plant that is colonized only by very specialized bacteria and therefore acts as a
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33 460 low-diversity reservoir from which the insects ingest bacteria (Colman *et al.* 2012; Jing *et al.* 2014;
34 461 Overholt *et al.* 2015).

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36 462 For most species there is no difference in the alpha-diversity parameters between ‘*Ca. P.*
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38 463 *solani*’ infected and non-infected specimens, indicating that the presence of the pathogen does not
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40 464 lead to a major change in the qualitative composition of the community. Still, for *E. incisus* and *E.*
41 465 *variegatus* a statistically significant increase was observed for all parameters in the infected
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43 466 specimens, compared to the non-infected.

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45 467 The analysis of abundance of the different taxa in the insect species in general revealed
46 468 microbial communities with low diversity, in which only a handful of genera were present with
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48 469 high abundance: *Bacillus* (Firmicutes), ‘*Candidatus Phytoplasma*’ (Tenericutes), *Cronobacter*
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50 470 (Proteobacteria), *Erwinia* (Proteobacteria), *Propionibacterium* (Actinobacteria), *Purcellliella*
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52 471 (Proteobacteria), *Rickettsia* (Proteobacteria), *Sodalis* (Proteobacteria), *Staphylococcus* (Firmicutes),
53 472 *Sulcia* (Bacteroidetes), and *Wolbachia* (Proteobacteria).

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55 473 The results obtained on the description of the bacterial microbiota of *E. incisus* and *P.*
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57 474 *alienus/confinis* agree with what was previously reported by Kobialka *et al.* (2018), who indicated a
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59 475 microbial community dominated by *Sulcia* for these species.
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3 476 Regarding *H. obsoletus*, our results that highlighted the presence of the genera *Sulcia*,
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5 477 *Wolbachia*, and *Purcellliella* confirming ~~data-the results-~~ previously obtained by Bressan *et al.*
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7 478 (2009) and Gonella *et al.* (2011) in northern Italy, but not ~~with~~ those obtained by Iasur-Kruh *et al.*
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9 479 (2017) in Israel. This latter study determined, using both classical and molecular microbiology
10 480 methods, that the genus *Sulcia* was the most abundant in *H. obsoletus*, followed by *Pectobacterium*.
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12 481 These differences may be explained by several variables, such as the different techniques used, and
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14 482 the different geographical areas from which specimens were sampled (~~northern Italy and Israel~~),
15 483 which leads to different climatic conditions and insect diet.

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17 484 The results obtained on *C. viridis*, with a high abundance of the genera *Sulcia* and *Sodalis*
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19 485 are in accordance to those previously reported by Michalik *et al.* (2014). Intriguingly, tThese results
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21 486 also revealed that *C. viridis* has a unique microbiota compared to the other insect species analyzed:
22 487 it has a more diverse composition, in which five different genera have a relevant level of
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24 488 abundance, and it's the only species in which we find a high abundance of the genera *Cronobacter*
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26 489 and *Sodalis*. These results suggest that either the higher diversity, leading to a more resilient
27 490 bacterial community, or these specific genera of bacteria could play a role in determining the non-
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29 491 vector status of this insect. Further studies will be conducted to determine if these elements can
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31 492 indeed be important and relevant for the development of an MRM strategy to reduce the spread of
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33 493 Bois noir.

34 494 The results regarding the beta-diversity in each analyzed insect species, infected and non-
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36 495 infected, highlighted the presence of two different groups among the insect species: (i) insects for
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38 496 which the presence or absence of the phytoplasma did not cause a major restructuring of the
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40 497 bacterial community, including the species *C. viridis*, *D. europaea*, *H. obsoletus*, and *P. spumarius*;
41 498 and (ii) insects for which the presence of the phytoplasma, not related to its abundance, caused a
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43 499 major change in the bacterial community, including the species *E. incisus*, *E. variegatus*, and *P.*
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45 500 *alienus/confinis*. Interestingly, among the analyzed species, these three are the only ones belonging
46 501 to the subfamily Deltocephalinae. The microbiota associated with members of this subfamily is
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48 502 usually characterized by the presence of two ancient mutualistic endosymbiotic bacterial genera:
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50 503 *Sulcia* and *Nasuia* (Kobialka *et al.* 2018). However, several studies reported that the symbiotic
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52 504 systems of Deltocephalinae leafhoppers can be very diverse, driven by processes of symbiont
53 505 acquisition and replacement, which can include both bacteria and fungi (Nishino *et al.* 2016;
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55 506 Brentassi *et al.* 2017; Kobialka *et al.* 2018; Mao and Bennett 2020). In our datasets, no OTUs
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57 507 assigned to the genus *Nasuia* were detected. This result is not in accordance with what is reported
58 508 by Kobialka *et al.* (2018), which found *Nasuia* in *E. incisus* and *P. alienus/confinis*. On the other
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60 509 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 Deltoccephalinae) (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 Deltoccephalinae), 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, 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 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 reinforces~~ 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 *Purcellliella*. 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

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3 544 several possible explanations: the first is that the interaction between the phytoplasma and other
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5 545 members of the microbiota lead to a rise of secondary mutualists, disadvantaging the primary
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7 546 mutualists such as *Sulcia* (Heddi *et al.* 1998); a second hypothesis is related to the host's immune
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9 547 response: it was demonstrated by Galetto *et al.* (2018) that the insect, *E. variegatus* in that study,
10 548 can activate a strong immune response when interacting with a phytoplasma that is not the one that
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12 549 it usually transmits. This immune reaction could change the bacterial community inside the host
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14 550 drastically, favoring more resistant bacteria, in particular Gram-positive species, as is seen in our
15 551 study for *E. incisus* and *E. variegatus*. A third hypothesis is based on results obtained of *D. citri* and
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17 552 '*Candidatus Liberibacter asiaticus*' by Vyas *et al.* (2015): this study demonstrated that the
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19 553 phytopathogen could modulate free amino acids availability by interfering with hexamerin storage
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21 554 pathways by regulating expression of amino acid storage protein genes. Such evidence suggests that
22 555 the reason why there is a dramatic reduction in genus *Sulcia*, which is heavily committed to amino
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24 556 acid production and encodes enzymes for synthesis of all amino acids required as animal nutrients,
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26 557 is simply due to the fact that an infected insect does not need such a high abundance of this bacterial
27 558 genus. On the other hand, sap-feeding insects rely heavily on the contribution of their obligate
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29 559 symbionts to maintain their metabolism (McCutcheon and Moran 2007). For this reason, the loss of
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31 560 dominance by the beneficial *Sulcia* endosymbionts could instead prove to be detrimental to the
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33 561 insect's fitness. More data on the fitness of the infected and non-infected insects would be needed to
34 562 give a correct interpretation of this result.

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36 563 Genus *Wolbachia* tended to be present only in the non-infected specimens and was
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38 564 ~~completely eliminated~~largely reduced in the infected insect species, except in the case of (i) *H.*
39 565 ~~*obsoletus*, in which it was still present but with lower abundance; and (ii) *D. europaea*, in which the~~
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41 566 abundance of *Wolbachia* was higher in the '*Ca. P. solani*' infected group ~~than the non-infected~~
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43 567 ~~group. Similar results were obtained by Fagen *et al.* (2012) who reported that the '*Ca. Liberibacter*~~
44 568 ~~*asiaticus*' titer within the insect was found to have a strong positive relationship with *Wolbachia*~~
45 569 ~~endosymbiont. Also, in this case,~~From these results, it becomes evident that the interaction is not
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47 570 just between the phytoplasma and *Wolbachia*, but that the insect species and the rest of the
48 571 microbiota play a role in determining ~~whether the abundance of *Wolbachia* is increased or reduced~~
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50 572 ~~upon infection~~its outcome. Still, considering that co-presence of phytoplasma and *Wolbachia* was
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52 573 not observed in the majority of the insect species, in general it is reasonable to conclude that a
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54 574 negative interaction governs the relationship between phytoplasma and *Wolbachia*. It should be
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56 575 established whether phytoplasma infection affects the *Wolbachia* concentration or if the presence of
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58 576 *Wolbachia* confers protection either by reduction in pathogen load, or competition with the
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60 577 pathogen (Krstić *et al.* 2018).

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3 578 The general reduction in the abundance of *Wolbachia* in phytoplasma-infected specimens suggests
4 that the phytoplasma can protect the insect from the possible detrimental effects of this intracellular
5 579 bacterium (feminization, male killing and a sperm-egg incompatibility) and might make *Wolbachia*
6 580 an unsuitable target for the development of an MRM strategy.
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12 583 The general reduction in the abundance of *Wolbachia* in phytoplasma-infected specimens suggests
13 that the phytoplasma can protect the insect from the possible detrimental effects of this intracellular
14 584 bacterium (feminization, male killing and a sperm-egg incompatibility) and might make *Wolbachia*
15 585 an unsuitable target for the development of an MRM strategy.
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20 588 **CONCLUSION**

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22 589 This study described the bacterial communities associated with seven insect species hosting ‘*Ca. P.*
23 *solani*’ and found in vineyards in North Italy. The mutualistic endosymbiont *Sulcia* was found as
24 590 the prevalent member of the microbiota in all insect individuals non-infected by the phytoplasma.
25 591 The non-vector *C. viridis* carries unique bacterial signatures (i.e, *Sodalis*, *Cronobacter*)
26 592 distinguishing its microbiota from that of vector insects, including its fellow xylem-feeder *P.*
27 593 *spumarius*.
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31 595 Beta-diversity analysis revealed that the xylem-feeding behavior of *C. viridis* and *P. spumarius*
32 596 gave a more similar structure in their microbiota in comparison with those of the phloem-feeding
33 597 vectors. Anyway, the aforementioned unicity of *C. viridis* reinforces the hypothesis that microbiota
34 598 elements could influence the vector / non-vector status of phytoplasma host insects.
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37 600 Analyses highlighted that, in North Italy, phytoplasma infection (not related to its abundance) is
38 601 associated with major change due to an increase of diversity in the microbiota structure exclusively
39 602 in *E. incisus*, *E. variegatus*, and *P. alienus/confinis*, the only species, among the analyzed ones,
40 603 belonging to the subfamily Deltocephalinae.
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43 605 Considering the specific bacterial genera on which our study focused (*Sulcia*, *Wolbachia*, and
44 606 *Dyella*), obtained data showed that ‘*Ca. P. solani*’ may have an adverse effect on the presence of the
45 607 obligate endosymbiont ‘*Ca. Sulcia muelleri*’ as well as the facultative endosymbiont *Wolbachia*,
46 608 while *Dyella* was not found. Further studies are necessary to elucidate whether observed differences
47 609 (reduction of *Sulcia* and *Wolbachia*, and increase of bacterial diversity) in phytoplasma infected
48 610 insects are associated with fitness increase or decrease.
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52 612 The results of this study indicate an interesting perspective regarding the microbial signatures that
53 613 could be relevant to determine whether an insect species can be a vector or not, opening up new
54 614 avenues for developing MRM-based approaches to contain BN spreading.
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613 '*Ca. P. solani*' may have an adverse effect on the presence of the obligate endosymbiont '*Ca. Sulcia*
614 *muelleri*' as well as the facultative endosymbiont *Wolbachia*.

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

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

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631 632 CONFLICT OF INTEREST

633 None declared.

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