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A bud's life: Metabarcoding analysis to characterise hazelnut big buds microbiome biodiversity

Silvia Turco [a,*], Federico Brugneti [a], Irene Giubilei [a], Cristian Silvestri [a], Miloš Petrović [b], Mounira Inas Drais [a], Valerio Cristofori [a], Stefano Speranza [a,c], Angelo Mazzaglia [a], Mario Contarini [a], Luca Rossini [d,*]

[a] Dipartimento di Scienze Agrarie e Forestali, Università degli Studi della Tuscia, Via San Camillo de Lellis snc, Viterbo 01100, Italy
[b] Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, Novi Sad, Serbia
[c] Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET-UNLP), La Plata, Argentina
[d] Service d'Automatique et d'Analyse des Systèmes, Université Libre de Bruxelles, Av. F.D. Roosevelt 50, Brussels 1050, Belgium

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ABSTRACT

Despite *Corylus avellana* L. being an economically important shrub species known for its resilience to adverse environmental conditions, it constantly faces attacks from a plethora of biotic entities. Among these, the mite pest *Phytoptus avellanae* is gaining importance, causing economic losses every year. This mite colonises the new generative and vegetative buds, leading them to become swollen and reddish, and drastically reducing hazelnut production. The biology behind gall formation is still poorly understood. This study provides a qualitative and quantitative description of the microbiome in both healthy and infested buds of two economically important hazelnut cultivars through metabarcoding of fungal ITS and bacterial 16 S. Potentially pathogenic genera such as *Fusarium* and *Pseudomonas* were predominant in the infested buds, along with the obligate intracellular bacterial genus *Wolbachia*. *Akanthomyces muscarius* was instead isolated from culture-based methods only from the infested buds. These findings could improve the understanding of gall ecology, supporting the management of mite populations, and they could also serve as a milestone for further studies on low-impact, monitoring-driven, and genetically targeted control strategies.

1. Introduction

In recent years, crops have faced increasing pressure from pests and diseases, leading to substantial economic damages and additional costs for farmers. This phenomenon is driven by several factors, including the introduction of alien species through global trade and climate change, which is causing the resurgence of native pests and diseases (Dutcher, 2007). As a result, farmers have increased the use of agrochemicalss for pest control purposes. Consequently, restrictive national and regional regulations on pesticide use in many areas worldwide have led the scientific community to seek alternatives (Bono Rosselló et al., 2023; Lee, 2019). Low-impact and environmentally friendly strategies typically rely on agronomic practices or natural enemies such as predators, parasitoids, or entomopathogenic bacteria, fungi, or viruses (Deka et al., 2021; Koller et al., 2023; Van Lenteren et al., 2018). In this way of farming, a comprehensive understanding of the interactions among the organisms in agroecosystems is crucial for enhancing yields while practising sustainable agriculture.

Hazelnut is an example of a profitable crop endangered by a growing set of biotic adversities (Turco et al., 2022, 2021). *Corylus avellana* L., a shrub species native to Mediterranean basin belonging to the Betulaceae family, is cultivated worldwide for its nuts, highly requested by food industries and consumers. High demand by the market has led to an expansion of hazelnut cultivation beyond the Mediterranean basin, towards countries such as the US, Chile, and Australia, where climate conditions are close to its place of origin (Ferrucci et al., 2023). With an average production above115,000 tons per year, Italy is the second-largest world hazelnut producer, after Turkey ("ISTAT," 2023, accessed on 11 June 2024).

Hazelnut is historically acknowledged for its resilience to adverse environmental conditions and biotic stressors; however, in the last few years a plethora of pathogenic agents belonging to *Fusarium,*

* Corresponding authors.
E-mail addresses: silvia.turco@unitus.it (S. Turco), luca.rossini@ulb.be (L. Rossini).

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Stemphylium, Piggotia, Erysiphe, and *Pseudomonas* genera (Ak et al., 2019; Bosco et al., 2018; Drais et al., 2023a; 2023b; Mazzaglia et al., 2023; Speranza et al., 2023) or insects such as the brown marmorated stink bug (*Halyomorpha halys* Stål) have become a persistent threat in several highly productive areas (e.g., Italy, Turkey, Georgia, and the US).

Climatic changes are endorsing the resurgence of an additional phytosanitary issue beyond pathogens and insects, the hazelnut mite pest *Phytoptus avellanae* (Nalepa)(Acari: Phytoptidae) *lato sensu* (Özman and Toros, 1997; Stamenkovic et al., 1997). Plants infested by this pest are identifiable by the presence of "big buds" or "pseudo galls" - vegetative and generative buds that have lost their viability, becoming swollen and reddish in colour (Özman and Toros, 1997; Westphal and Manson, 1996). These enlarged buds serve both as a food source and shelter for the mites that develop within, often remaining protected from conventional phytosanitary treatments.

The umbrella name *P. avellanae* includes two cryptic mite species, one developing within buds and responsible for gall formation, and a second one with a vagrant lifestyle, mostly present on the abaxial side of the leaves (Cvrković et al., 2016). According to the current literature, migration is mainly due to wind, given the small size and morphology of the mite. During spring, nymphs migrate to new generative and vegetative hazelnut axillary buds, where they feed and reproduce until the following spring (De Lillo et al., 2018).

The symptoms of the infested buds are easily noticeable during winter, when leaves are absent, and in the blooming phase during the early spring, when leaflets start opening. The mite infestation is affecting the development of both vegetative and generative organs, including branches, leaves, flowers, and nuts (Lippi et al., 2022). To date, the biology and epidemiology of these pests are still not fully understood, with various hypotheses regarding the formation of the big buds (Gatjens-Boniche, 2019; Giron et al., 2016). The involvement of bacteria in gall and pseudo-gall formation is well-documented (Harris and Pitzschke, 2020) and recent evidence has supported the hypothesis of a symbiotic relationship between gall-inducing mite species and bacteria (Klimov et al., 2022). Understanding the ecology and biology behind the hazelnut's big bud formation, whether endogenic (mite-induced) or exogenic (involving symbiotic relationships), is crucial for the formulation of proper mite control strategies. Despite its importance, the existing literature provides only fragmented insights into the galls' microbiome and the microorganisms interactions. This gap in knowledge is not exclusive to *P. avellanae*, as similar studies are lacking for other bud infesting mite species as well. Therefore, analysing the differences in microbiomes between infested and non-infested buds is an essential initial step, laying the groundwork for further research.

This study aimed to explore buds and big buds from Tonda Gentile Romana and Tonda di Giffoni, two of the most economically important cultivars of hazelnut in Italy. Metabarcoding analysis of both ITS and 16 S regions was conducted to compare the fungal and bacterial microbiomes of healthy and mite-infested buds, along an *in silico* functional microbiome prediction and a culture-based isolation of bacterial and fungal species. Our findings provide additional insights into *P. avellanae* and the ecological microenvironment influencing its invasive process. Importantly, this research sets the stage for future studies aimed at developing low-impact, monitoring-driven, and genetically targeted control strategies for hazelnut mite pests.

2. Materials and methods

2.1. Sampling

Buds sampling was carried out in February 2023, in the collection orchard "Le Cese", established in 2000 in a relevant hazelnut-growing area surrounding Vico Lake (latitude 42°20'54'' N; longitude 12°11'38'' E; altitude 570 m a.s.l.) in Central Italy. In this non-treated collection field, each of the 48 hazelnut cultivars is represented by 3 multi-stemmed plants spaced 4 ×5 m and trained as an open vase. The orchard is equipped with a sub-irrigation system, managed with standard orchard techniques that do not include any use of agrochemicals, and characterised by a Mediterranean climate, typical for hazelnut production. Plants are deployed on sandy-clay-loam soil (according to the classification of US Department of Agriculture) with pH 6.1, total organic matter 2.1 % and low total calcium content (7.4 g kg^{-1}).

During our recent monitoring we noticed that two widely cultivated and economically important hazelnut cultivars showed different susceptibility to mite infestation. For this reason, they were selected for the analysis: Tonda Gentile Romana (TR), showing less susceptibility, and Tonda di Giffoni (TG) showing more susceptibility. For a representative random sampling, healthy-looking and swollen-infested buds were collected from three different plants per cultivar (named A, B, and C) and from at least three different branches per plant.

2.2. DNA extraction and Illumina sequencing

The healthy and the swollen mite-infested buds (Fig. 1) from each cultivar were surface sterilised in 2 % NaOCl for 2 min and rinsed three times with sterile distilled water (SDW). After sterilisation, 10 buds per each replicate (A, B, and C trees) and per each cultivar were pooled together and ground in liquid nitrogen with mortar and pestle. One hundred mg of the lyophilized powder was used for total DNA extraction using a CTAB protocol (Saito et al., 2013) and five technical replicates were carried out. Thirty µl of total DNA, with a concentration of at least 10 ng/µl, were sent to Eurofins Genomics (Konstanz, Germany) for amplification and Illumina MiSeq sequencing of the hypervariable V1-V3 regions in the bacterial 16 S rRNA gene and of the ITS1 fungal Internal Transcribed Spacer (ITS) gene. Sequencing adapters and primers were removed by Eurofins Genomics using Cutadapt v.4.5 (Martin, 2011), a quality check was carried out with *fastp* v0.23.2 (Chen et al., 2018), while paired-end reads were merged using the software Flash v.1.2.11 (Magoč and Salzberg, 2011).

2.3. Bioinformatic and statistical analysis

The bioinformatics analysis was carried out using the Quantitative Insight into Microbial Ecology 2 (QIIME2) pipeline (v.2023.2.0) within a Docker container (Bolyen et al., 2019). Merged (forward R1 and

Fig. 1. Hazelnut twigs showing mite-infested buds. A) Detail of buds on hazelnut branch; B) Section of healthy hazelnut buds (on top) compared to the swollen and reddish mite-infested buds (bottom); C) Mite infested buds (left) compared to the healthy buds.

reverse R2) reads were demultiplexed, chimaera filtered, and denoised through the Divisive Amplicon Denoising Algorithm (DADA 2). The obtained feature table with the amplicon sequence variants (ASVs) and the representative sequences were used for downstream analysis. Fungal taxonomic classification was carried out using the *sklearn* algorithm and a custom database built with the *rescript* QIIME2 plugin (Robeson et al., 2021) using the NCBI Fungi RefSeq ITS project (PRJNA177353) and the *Corylus avellana* L. sequences (taxID 13451). The bacterial taxonomic classification, instead, was carried out using the *sklearn* algorithm combined with the SILVA 138 database. MAFFT and FastTree implemented within the QIIME2 workflow were used to build a phylogenetic tree of the representative sequences. ASVs classified as hazelnut, chloroplast, mitochondria, unclassified ASVs and low-frequency ASVs were discarded, according to Bokulich et al. (2013). The filtered ASV table, the taxonomy table, the representative sequences, the phylogenetic tree, and the metadata file with sample descriptions were then imported within the R environment (v.4.2.3), and further processed with the *phyloseq* package (v1.42.0) (McMurdie and Holmes, 2013). Sequences were rarefied using a sample size of 885 reads for the fungi and 889 reads for the bacteria, with refraction curves that nearly reached a plateau (data not shown). Shannon and Simpson indexes were used for alpha within-sample diversity analysis, whilst richness was evaluated through the Chao1 and ACE indexes. Statistically significant differences between sample state and among the two cultivars were carried out through a Kruskall-Wallis test paired with a Dunn *post-hoc* test.

Beta diversity between samples was evaluated through a non-metric multidimensional scaling (NMDS) and a Principal Coordinates Analysis (PCoA) based on Bray-Curtis diversity. Correlation between the microbial diversity, cultivars, and state of the infestation was further tested through the permutational multivariate analysis of variance (PERMANOVA, 1000 permutations), considering $\alpha = 0.05$ as a threshold for significance. Relative abundance was plotted explicitly for the 20 most abundant genera, while the remaining groups were enclosed in the "<1" category. Differences in microbial abundance between healthy and infested buds were evaluated using DESeq2 package (v1.38.3) (Love et al., 2014), with the Wald test, a parametric fit, and an adjusted *p* value of 0.05.

Functional guilds classification of the identified fungal ASVs was carried out using the *fungaltraits* v 0.0.3 package within the R environment, which combines both the FunGuild and the Fun^{Fun} databases (Põlme et al., 2020). Bacterial ASVs were instead functional classified using the *picrust2* plugin within QIIME2, and the *ggpicrust2* R package (Douglas et al., 2020; Yang et al., 2023), with DESeq2 method for differential analysis; the adjusted *p* value set at 0.05, and the KO (KEGG Orthology), KEGG EC and MetaCyc for pathway annotation.

2.4. Sample processing for culture-based isolation

After the application of the same sterilisation process described in 2.2, six buds per tree (3 healthy and 3 infested) from each cultivar were sectioned with a sterile scalpel. Half of the section was plated on Potato Dextrose Agar (PDA) plate for fungal isolation, while the other half was plated onto Yeast Extract Agar (YEA) plates for bacterial culture, for an overall 18 plates per cultivar and, thus 36 plates in total. The plates were incubated at 25 °C for fungal growth and at 28 °C for bacterial growth, respectively. Mixed colonies were further re-isolated to obtain a single pure culture. Bacteria were identified by Sanger sequencing after a colony PCR using the ribosomal rRNA 16 S primers 27 F-1429R (Lane, 1991) with the following PCR program: 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, 72 °C for 5 min. Fungal DNA was extracted from mycelium using the standard CTAB method (Saito et al., 1981) and the molecular characterization was carried out by amplification of the internal transcribed spacer (ITS) with ITS1-ITS4 primers (White et al., 1990) using the following PCR program: initial denaturation at 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, final elongation 72 °C for 5 min.

3. Results

3.1. Sequencing and DADA2 denoise output

Fungal ITS1 amplicon sequencing produced around 200k reads per sample, which, after demultiplexing, denoising, and chimaera filtering, yielded a total of 395 Amplicon Sequence Variants (ASVs), shown in Table S1. The bacterial 16 S amplicon sequencing, instead, produced 172–382k reads per sample, which, after demultiplexing, denoising, and chimaera filtering, yielded a total of 754 ASVs (Table S1). After removal of the ASVs related to the host, chloroplasts, mitochondria, unassigned, and after removal of low frequency ASVs (we kept only those represented by at least 500 reads and that were present in at least 10 % of the samples), 41 taxa were kept for the ITS profiling and 33 taxa for the 16 S profiling.

3.2. Alpha and beta diversity of microbial communities

The alpha (within-sample) diversity of the fungal community, expressed by Shannon and Simpson indexes, showed a comparable heterogeneity among healthy and mite-infested buds, although the boxplot shows an overall higher variability for healthy buds compared to infested ones (Figure S1-A). The richness values, expressed by Chao1 and ACE indices, were slightly lower in infested buds (Figure S1-A). Conversely, bacterial communities showed higher abundance (higher richness) in the infested buds (Figure S1-B). The boxplot in this case showed an overall higher variability in the infested buds than in the healthy ones (Shannon and Simpson indexes), while the Chao 1 and ACE had median values apparently far from each other. No significant differences were found in the richness between the healthy buds.

Alpha diversity per cultivar is reported instead in Figure S2A and S2B. The fungal community reported similar levels and higher variability for the Shannon and Simpson indexes, while the lower variability and the highest gap between the median values have been observed for the Chao1 and ACE indexes (Figure S2A). Significant differences were found only in the community richness between the cultivars evaluated with the Chao1 index ($p = 0.026$).

A different scenario was observed for the bacterial community, where the median values were almost comparable, between the two cultivars, for the Chao1 and ACE indexes, while a wide gap between the medians was observed for the Shannon and Simpson indexes. Overall, the bacterial community showed almost the same variability, expressed by the width of the boxes.

PCoA and NMDS analyses of the fungal beta diversity based on Bray-Curtis distance highlighted a clear distinct pattern between the healthy and the infested buds from both the statistical tests adopted to analyse this part of the dataset (PERMANOVA, $F = 3.82$, $R^2 = 0.27$, $p = 0.001$) (Fig. 2). A slightly different scenario was assessed for the differences among the two hazelnut cultivars, given that the PERMANOVA test indicated a significant difference, even if closely below the threshold of $p = 0.05$ (PERMANOVA, $F = 2.29$, $R^2 = 0.16$ and $p = 0.024$). As for the fungal community, statistically significant differences were found between the cultivar bacterial community richness ($p = 0.05$) and in the Simpsons within-sample diversity between the two cultivars ($p = 0.016$).

The dispersion of the bacterial community beta diversity between healthy and infested buds was distinct according to the PERMANOVA test, which indicated a significant difference just below the threshold of $p = 0.05$ (PERMANOVA, $F = 5.01$, $R^2 = 0.18$, $p = 0.044$). The differences between the cultivars, instead, were confirmed by a significant PERMANOVA test ($F = 13.93$, $R^2 = 0.49$, $p = 0.001$).

3.3. Relative abundance of fungal communities

At the phylum level, the fungal community was mostly represented by Ascomycota (75–95 %), with a lower relative abundance of Basidiomycota (15–25 %), which increase drastically in two infested

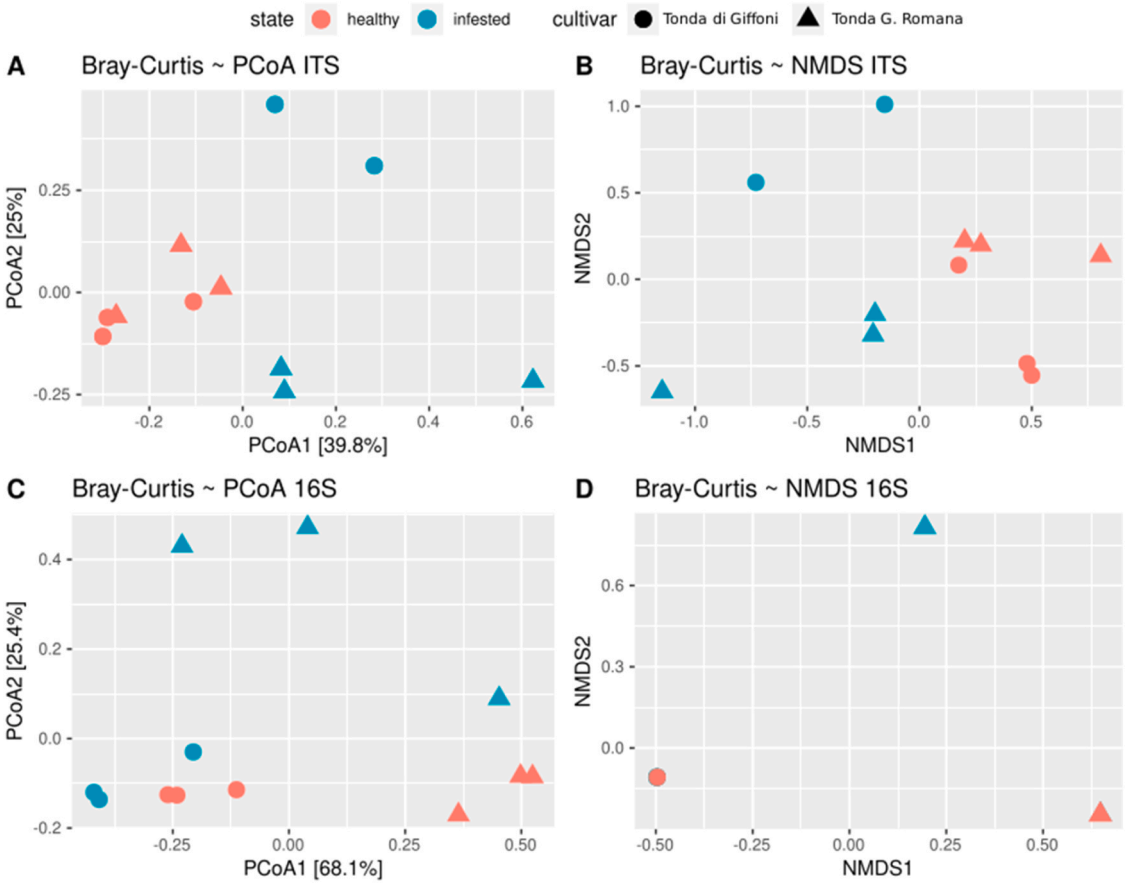

Fig. 2. PCoA and NMDS of the fungal (A, B) and bacterial (C, D) community beta diversity between healthy (red) and infested buds (turquoise) of the two cultivars Tonda di Giffoni (dot) and Tonda Gentile Romana (triangle). Note that in D) the points coordinates are overlapping.

samples of the cultivar Tonda di Giffoni (36Bi, 36 Ci) (Figure S3, Table S2). At the genus level, *Ramularia* (Dothideomycetes, Mycosphaerellaceae) was the most abundant in the healthy buds (from 32.7 in 9As to 82 % in 36As), regardless the hazelnut cultivars, followed by *Didymella* (Dothideomycetes, Didymellaceae), especially in the Tonda Gentile Romana cultivar (from 21 % in 9Cs to 53 % in 9Bs) and *Fusarium* (Sordariomycetes, Nectriaceae) (1.3 % in 9Bs to 22.4 % in 36Cs) (Fig. 3). The basidiomycete *Vishniacozyma* (Tremellomycetes, Bulleribasidiaceae) was present in all the healthy buds, ranging from 4 % in 9Bs to 22.7 % in 36Cs. *Cladosporium* (Dothideomycetes, Cladosporiaceae) abundance in the Tonda di Giffoni cultivar was below 1 %, while slightly more abundant in Tonda Gentile Romana, with 1.35 % in 9As and reaching 8.36 % in 9Cs. Among the less abundant ("<1"), it is worth mentioning *Cercospora, Papiliotrema, Alternaria, Taphrina,* and *Elsinoe*.

Interestingly, the relative abundance of the fungal community changed in infested buds. *Ramularia* drastically decreased in the Tonda di Giffoni cultivar (from 3 % in 36 Ci to 11.74 % in 36Ai), while it increased in the Tonda Gentile Romana cultivar (~47 % in 9Ai and 9 Ci). *Fusarium* became the most abundant in Tonda Romana cultivar, by being 37 % in 9Ai and reaching the maximum abundance of 93.3 % in 9Bi (Fig. 3). *Cladosporium* increased in Tonda di Giffoni (from 7.18 % in 36Bi to 27.95 % in 36Ai), while it almost disappeared in the Tonda Romana cultivar (<0.52 %). *Acremonium* (Hypocreales family) increased in the Tonda Gentile Romana (from 2.13 % in 9 Ci to 4.53 % in 9Bi). *Didymella* was drastically reduced in the Tonda Gentile Romana and increased only in Tonda di Giffoni 36Bi by 5.34 %.

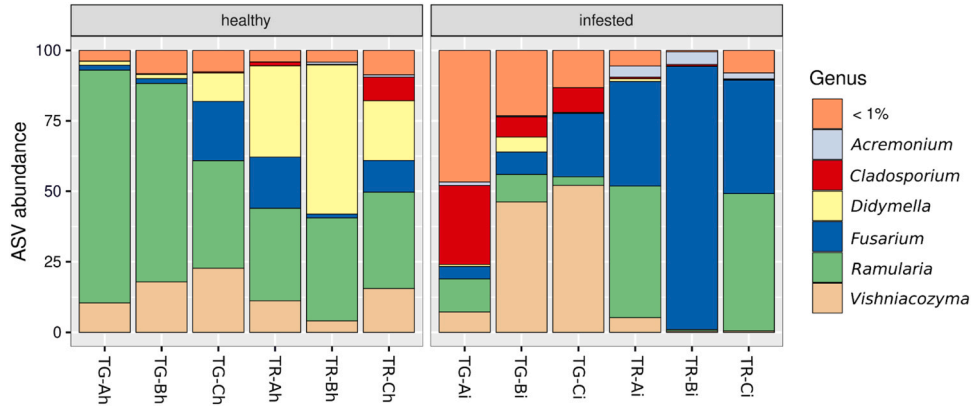

Fig. 3. : Relative abundance of fungal community in healthy (h) and infested (i) buds of Tonda di Giffoni (TG) and Tonda Gentile Romana (TR) cultivars.

3.4. Relative abundance of bacterial communities

Proteobacteria was the predominant phylum among the bacterial community (up to 98 %), followed by Actinobacteriota, Bacteroidota and Firmicutes (Figure S4, Table S3). *Pseudomonas* was the predominant genus in the Tonda di Giffoni cultivar, spanning from 53 % (36Cs) to 66.55 % (36Bs) in the healthy buds and increasing from 55 % (36 Ci) to 79.2 % (36Ai) in the infested buds (Fig. 4). Compared to Tonda di Giffoni cultivar, *Pseudomonas* relative abundance in Tonda Gentile Romana was much lower in the healthy buds, from 1.96 % (9Cs) to 9.14 % (9Bs), with a clear increase in the infested buds, especially in sample 9Bi (41.28 %) and 9 Ci (20.88 %). *Sphingomonas* genus was predominant in Tonda Gentile Romana healthy buds (34–53 %), present in Tonda di Giffoni only from 4 % (36Bs) to 13.3 % (36Cs), significantly reduced in the infested buds (0.41 % in 36Ai to 16.7 in 9Ai). *Aureimonas* genus was abundant in the healthy buds as well, spanning from 8.75 % in 36Bs to 40.8 % in 9Cs, and decreased only in some of the infested buds. Relatively abundant in the healthy buds were the genera *Stenotrophomonas* (1–13.42 %), *Luteimonas* (1–5.63 %) and *Methylobacterium* (0.1–5.4 %). In the infested buds, *Stenotrophomonas* disappeared in Tonda Gentile Romana, while it remained abundant in Tonda di Giffoni (10–17.42). *Luteimonas* disappeared in Tonda di Giffoni 36Ai and 36Bi and Tonda Gentile Romana 9Bi and 9 Ci, while it increased in samples 36 Ci (4.83 %) and 9Ai (29.41 %). *Methylobacterium* was reduced in all the infested samples. Interestingly, the genus *Wolbachia* was abundant only in the infested buds, especially in Tonda Gentile Romana cultivar, were it ranged from 1.28 % in 36Ai to 50.84 % in 9 Ci.

3.5. Core microbiota of hazelnut buds

We investigated the common ASVs, including those with the lowest frequency, shared among hazelnut cultivars and different conditions. The core microbiota of the hazelnut buds, found across all cultivars and infestation state, consisted of eleven fungal ASVs (*Cladosporium, Aureobasidium*, *Cercospora, Ramularia, Didymella, Alternaria, Fusarium, Taphrina, Dioszegia, Vishniacozyma* and *Papiliotrema*) (Figure S5A) and nine bacteria genera (*Pedobacter, Methylobacterium, Allorhizobium, Aureimonas, Novosphingobium, Sphingomonas, Pseudomonas, Luteimonas* and *Xanthomonas*) (Figure S5B). *Aspergillus* and *Golubevia* were exclusively found in the infested buds, not in the healthy ones. Conversely, *Sarocladium* was present only in the healthy buds across both cultivars. Concerning bacteria, *Verticiella* and *Dyella* were present only in the healthy buds and not in the infested buds, while six genera were present in the infested buds and not in the healthy ones: *Corynebacterium, Roseomonas, Wolbachia, Schlegelella, Variovorax* and *Massilia. Neocucurbitaria, Neosetophoma, Biscogniauxia, Psathyrella, Cytospora* and *Bullera* were shared among Tonda di Giffoni buds, while *Radulidium, Phaseospheria* and *Akanthomyces* were shared among the Tonda Gentile Romana buds. *Flavobacterium* and *Erwinia* were common among Tonda di Giffoni buds, while *Frigoribacterium* was characteristic of Tonda Gentile Romana buds.

3.6. Differentially abundant bacterial and fungal genera

The statistical significance of differentially abundant microbial genera was evaluated between healthy and infested buds and between cultivar using DESeq2, the Wald test and a parametric fit, and visualised as a heatmap (Figs. 5A and 5B). Only a few fungal genera were significantly different in the healthy buds compared to the infested ones. *Papiliotrema* and *Phaeosphaeria* were enriched in the healthy Tonda Gentile Romana buds, while *Buckleyzyma, Acericola*, and *Erythrobasidium* were significantly abundant in the healthy Tonda di Giffoni samples. *Cercospora, Aureobasidium, Dioszegia, Alternaria, Ophiognomonia, Kondoa, Cladosporium, Papiliotrema, Golubevia, Bullera, Quixadomyces, Diplodia, Psathyrella, Biscogniauxia, Vishniacozyma,* and *Dothiorella* were significantly enriched in the Tonda di Giffoni infested buds, while *Fusarium, Elsinoe, Radulidium, Acremonium, Zymoseptoria, Taphrina, Parahyridaria,* and *Gibellulopsis* were enriched in the Tonda Gentile Romana infested buds. The rest of the genera shown in Fig. 5A resulted in depletion in either healthy or infested buds. *Corynebacterium, Paracoccus, Acinetobacter, Dechloromonas, Sphingobium,* and *Erwinia,* were enriched in the Tonda di Giffoni infested buds, while Tonda Gentile Romana infested buds were mostly enriched in *Roseomonas, Massilia, Bacillus, Cutibacterium, Schlegella, Pantoea, Hymenobacter, Variovorax* and *Wolbachia* (Fig. 5B). The healthy buds of Tonda Gentile Romana were mostly enriched in *Verticiella, Allorhizobium, Friedmanniella, Nocardioides, Dyella*, and *Brevundimonas. Stenotrophomonas, Flavobacterium, Hymenobacter*, and *Pseudomonas* were slightly enriched in the healthy buds of Tonda di Giffoni.

3.7. Functional analysis of microbial community

The ecological functional analysis was carried out to identify microbial communities through FungalTraits for the fungal classification and Picrust2 for the bacterial community. The FungalTraits database could not classify all the fungal taxa,nevertheless, most of the fungal genera (*Taphrina, Phomopsis, Penicillium, Phaeosphaeria, Neonectria, Fusarium, Diaporthe, Colletotrichum, Cladosporium, Cercospora,* and *Alternaria*) belonged to the "Plant Pathogen" category (Fig. 6). Some taxa belonged to different categories, such as *Aspergillus, Cladosporium, Exophiala*, and *Colletotrichum* or could be both saprotroph or endophyte (i.e. *Cladosporium*). The only Basidiomycota classified (*Psathyrella*) belonged to the wood saprotroph category (data not shown).

The putative role of bacterial communities was explored by

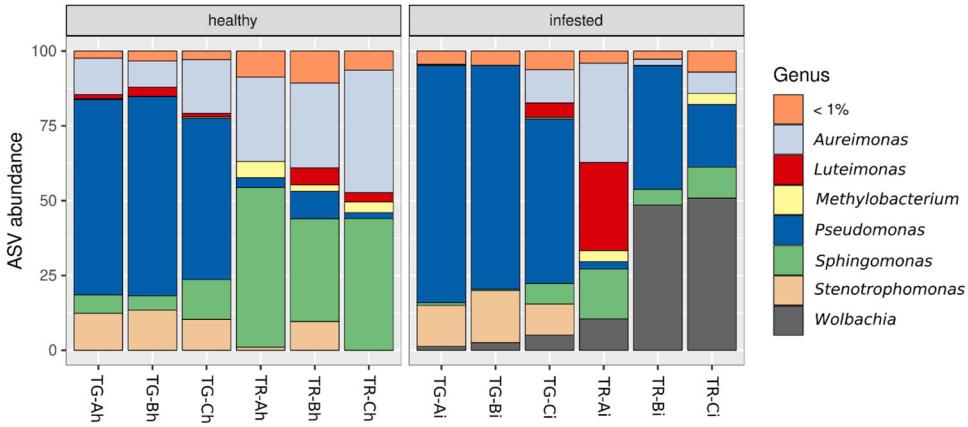

Fig. 4. : Relative abundance of bacterial communities in healthy (h) and infested (i) buds of Tonda di Giffoni (TG) and Tonda Gentile Romana (TR) cultivars.

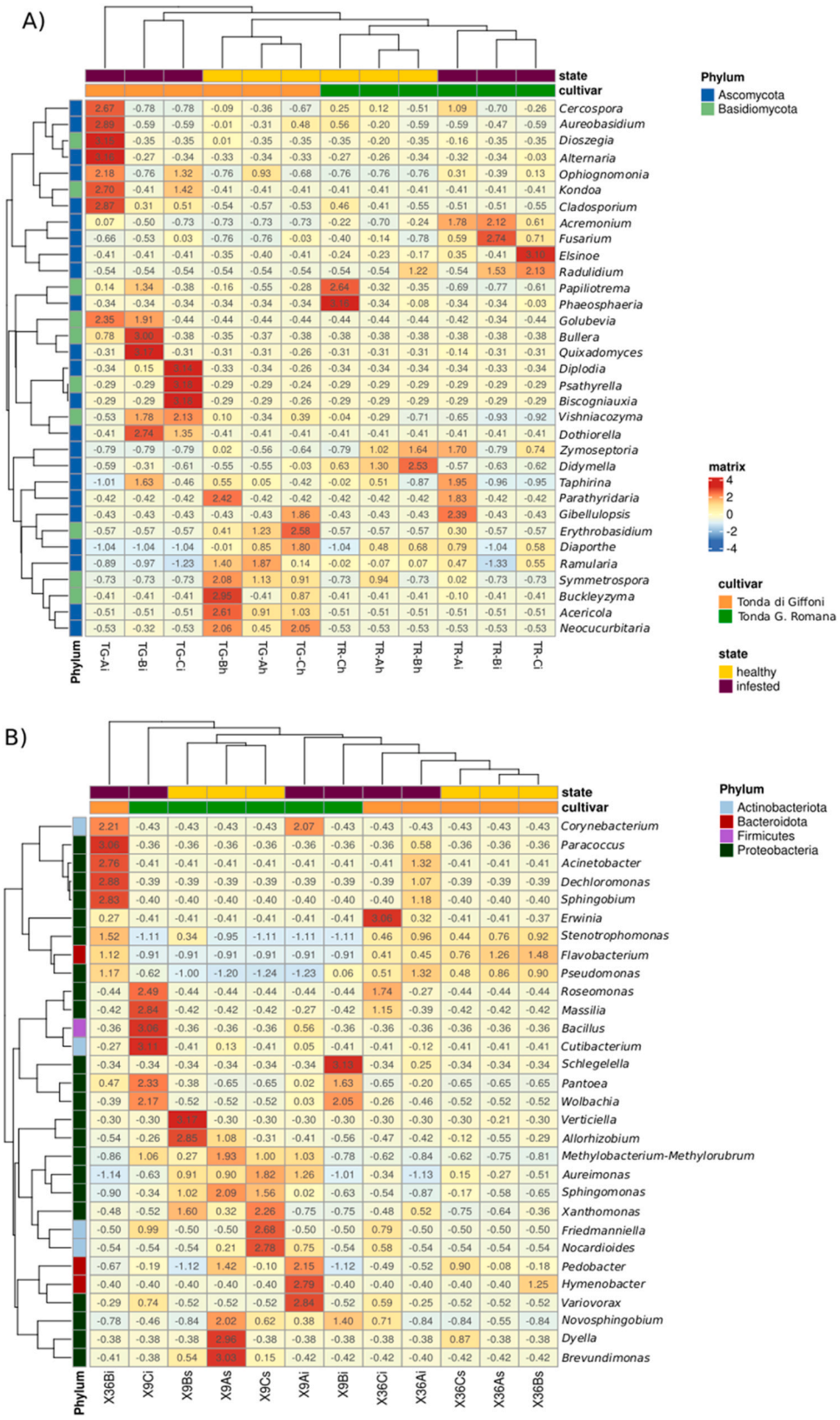

Fig. 5. : Heatmap showing the most significantly different genera: A) Fungal genera; B) Bacterial genera. The numbers within the cell indicate the log2 fold change calculated by DESeq2.

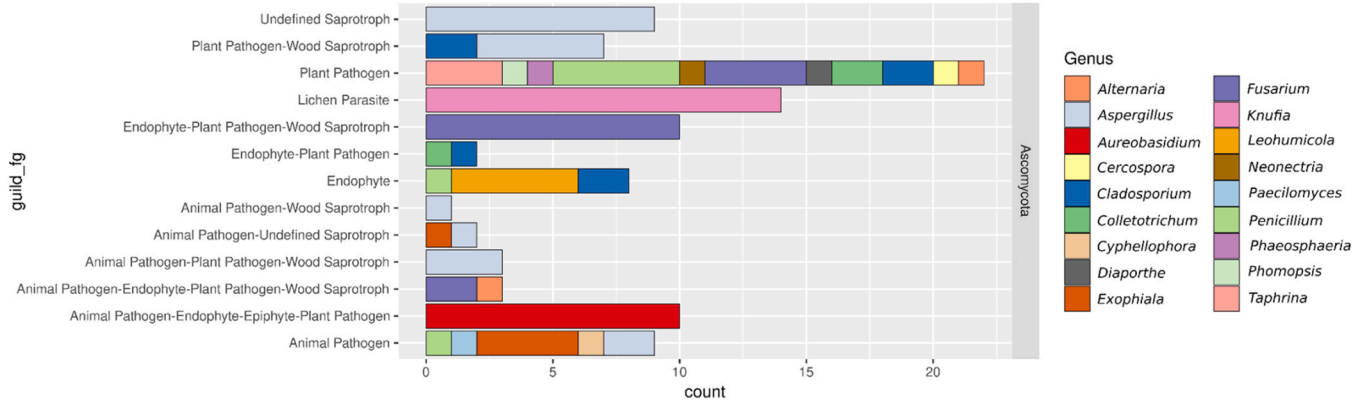

Fig. 6. : Functional analysis carried out through the FungalTraits database based on the FunGuild function.

statistical analysis of the 16 S abundance based on Picrust2 and the availability of the identified bacterial taxa in the KEGG KO database, the KEGG Enzyme database and the KEGG pathway database (Fig. 7). The most abundant KO categories were related to metabolic pathways such as carbohydrate metabolism (pyruvate, amino sugar, and nucleotide sugar), cofactor and vitamins (ubiquinone and other terpenoids), and amino acid metabolism such as glutathione. The barplot in Fig. 7 did not show high differences in the spread of KEGG categories between healthy and infected samples. Among the 30 most significant KEGG EC (EC number system) categories, it is worth mentioning a reduction in UDP-4-amino-4-deoxy-L-arabinose aminotransferase in the infested buds compared to the healthy ones, together with a reduction in mandelamide amidase and malate dehydrogenase (Figure S6A). The (S)-3-amino-2-methylpropionated transaminase and the thymidylate synthase (FAD) were instead slightly more abundant in the infested buds (Figure S6A). Among the 30 most significant KEGG pathway categories, the biosynthesis of histidine, purine and pyrimidine pathways were slightly more abundant in the healthy buds. Conversely, the pentose phosphate, the adenosine nucleotide degradation, L-isoleucine, and L-valine biosynthesis pathways were instead more abundant in the infested buds (Figure S6B).

3.8. *Identification of cultured bacterial and fungal isolates*

Culture-based isolation of both healthy and infested buds from the two different cultivars led to the identification of seven fungal genera and eleven bacterial genera (Fig. 8). The most abundant fungal species was *Akanthomyces muscarius,* isolated only from the infested galls (14 isolates), followed by *Fusarium* (13 isolates) and *Didymella corylicola* (11 isolates), isolated from both healthy and infested buds (Fig. 8A). When aligned on the NCBI nucleotide database usingBLASTn, the *Fusarium* sequences showed the highest percentage of similarity to *Fusarium lateritium*, *Fusarium tricinctum,* and *Fusarium tricinctum* species complex, in line with previous findings (Turco et al., 2021). Among the other isolates, *Sarocladium strictum* (2 isolates)*, Diaporthe* sp. (1 isolate)*, Alternaria* sp. (1 isolate) were detected only in the healthy buds, while *Biscogniauxia nummularia* was isolated only once from an infested Tonda Romana bud. The colony PCR on the bacteria plates allowed the identification only at the Genus level, resulting mostly in *Pseudomonas* (31 isolates)*, Xanthomonas* (3 isolates), *Frigoribacterium* (3 isolates), *Rhizobium* (3 isolates), *Stenotrophomonas* (3 isolates), *Pantoea* (2 isolates), and only one isolate of *Brevibacterium, Corynebacterum, Methylobacterium, Sphingomonas,* from both healthy and infested galls (Fig. 8B).

4. Discussions and conclusion

Phytoptus avellanae, with its feeding activity, is responsible of damages to generative and vegetative buds and induces physiological changes, such as a reduced photosynthetic activity, a disruption of normal plant growth processes, deformation, and necrosis of tissues (Özman and Toros, 1997). All these physiologic effects can have a significant impact on the quality and quantity of nut production, underlining the importance of effective management strategies, including integrated pest management approaches that incorporate biological, chemical, and agronomical methods. Understanding the intricate ecology of big bud formation and the relationship between hazelnut and the Eriophyoid mite is crucial for improving hazelnut production and safeguarding this economically important crop. Since the biological

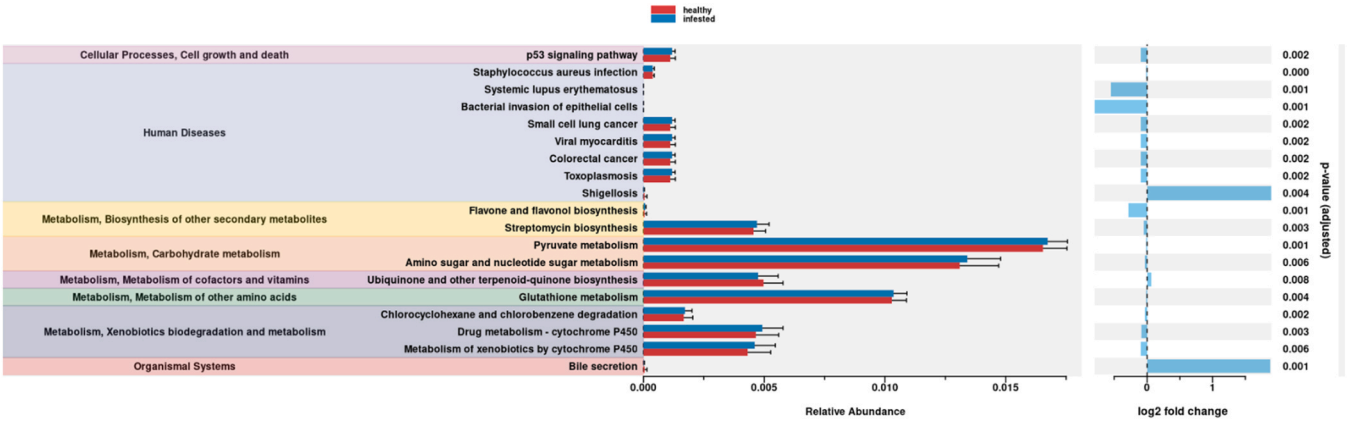

Fig. 7. : Picrust2 functional analysis showing the relative abundance (and its related standard error) of the most significant ($p < 0.05$) differentially abundant predicted KEGG KO categories.

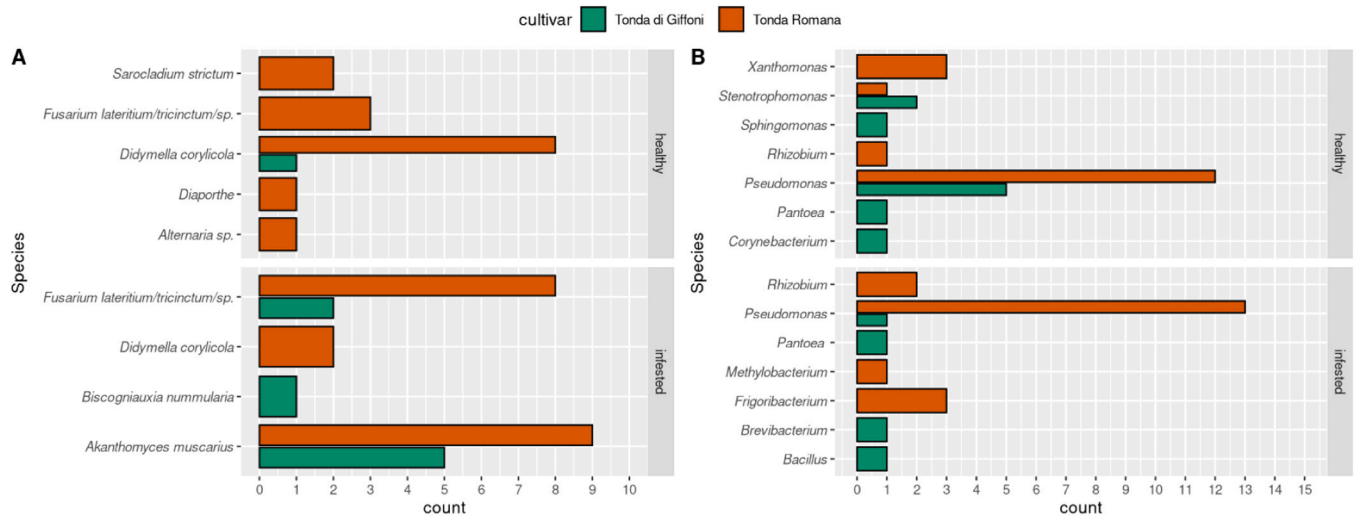

Fig. 8. : Absolute counts of the isolated fungal genera (A) and isolated bacteria genera (B) from the two cultivars, respectively.

mechanisms are still unclear, this work wanted to explore the intimate gall microbial populations and their possible involvement into the gall formation. Two different cultivars, Tonda Gentile Romana and Tonda di Giffoni, were chosen for their economic and cultural importance within the Italian hazelnut production. "Le Cese" collection field was suitable for a non-biased, representative sampling, where the mite infestation level depends mostly on the cultivar susceptibility rather than climate and orchard managements. In this regard, it has already been shown that irrigation methods do not interfere with penetration mechanisms and further colonisation of the buds by *P. avellanae* (Contarini et al., 2022). Tonda di Giffoni was more abundant in terms of fungal community, while the Tonda Gentile Romana showed a higher richness for the bacterial one. This first evidence may be explained by the already established levels of susceptibility to the mite, with the Tonda Gentile Romana being less susceptible when compared to other Italian cultivars (Contarini et al., 2022; Gantner, 2009; Rovira and Tous, 2001). *Phytoptus avellanae* infestation seemed to affect the bacterial and fungal abundance rather than their diversity. As an example, *Pseudomonas* and *Fusarium* drastically increased their presence in the infested buds and their association with specific hazelnut diseases is established (Santori et al., 2010; Turco et al., 2022, 2021; Vitale et al., 2011).

An interesting exception was *Wolbachia*, which was present only in the infested buds. This genus is an obligate intracellular bacterium that primarily infects arthropods and other invertebrates (Bourtzis and O'Neill, 1998; Weeks and Breeuwer, 2001) and its association with Eriophyoidea was already proven (Klimov et al., 2022). *Wolbachia* is generally considered an endosymbiont whose infection causes, in the host, alterations in reproductive processes that may include induction of parthenogenesis, death of males born from infected females, and cytoplasmic incompatibility which results in the death of embryos when *Wolbachia*-infected males mate with uninfected females or with females carrying a different strain of *Wolbachia* (Werren et al., 2008). Even if these bacteria are not reported to induce gall or big bud formation directly, they may manipulate cytokinin levels which, instead, are known to stimulate cell division and proliferation of plant tissues, resulting in the formation of neoplasms (Akhtar et al., 2019; Jameson, 2000). Therefore, although the consequences of the *P. avellanae* - *Wolbachia* association were not examined in detail in this study, it may be assumed that the bacterium is involved in inducing alterations in the mite's reproductive processes or plays a role in the mite's defence against other organisms. Ourry et al. (2021) observed that *Wolbachia* can cause alterations in host-associated bacterial communities, with increases and decreases in several taxa. In this study, the presence of *Wolbachia* was negatively correlated with *Sphingomonas* and *Aureimonas,* whose relative abundance was reduced in the infested galls and whose role in the ecological dynamics of *P. avellanae* or in the physiological reactions of the plant should be better understood in the future. In addition to *Wolbachia*, it is worth mentioning the presence of another species, the entomopathogenic fungus *Akanthomyces muscarius*. Its presence was not so evident or significantly abundant in the metagenomics results (or limited to the <1 categories, in Fig. 3), however this fungus was constantly isolated on PDA plates from the mite-infested buds. Its endophytic behaviour has been reported in wild context (*Acer campestre, Laurus nobilis, Myrtus communis, Nypa fruticans, Quercus robur*) and in crops (*Brassica oleracea, Cucumis sativus, Prunus cerasus*), and it has been extensively reviewed by Nicoletti and Becchimanzi (2020). Due to its proven ability as biocontrol agent against insect pests, an isolate of *A. muscarius* has been used for the preparation of bio-insecticides (Mycotal®, Verticillin®) to control whiteflies, thrips, aphids, and mites [Verticillin®, (Faria and Wraight, 2007)]. However, its association with mite-infested hazelnut buds has been already reported in the Viterbo area (Mazzaglia et al., 2023). All the fungal genera present in the core microbiome have been already identified as possible hazelnut endophytes and their ecological role recently reviewed (Nicoletti and Zimowska, 2023) with the only exception of *Papiliotrema,* a ubiquitous basidiomycetous yeasts (Liu et al., 2015). *Pseudomonas* spp. and *Xanthomonas*, are known to be associated with hazelnut diseases (Nicoletti et al., 2022; Turco et al., 2022), *Allorhizobium* is known to have plant growth promoting activities (Majeed et al., 2018), *Pedobacter* and *Sphingomonas* have been reported in mite-induced gall (Klimov et al., 2022). Some of the identified bacterial and fungal genera are known to be saprotroph, endophytes, potential plant pathogen, or plant growth promoting. However, it is worth pointing out that the results of the functional analysis presented in this study are limited to the information contained in the considered databases, namely FungalTraits or KEGG. Despite this apparent limitation, however, we showed a clear scenario of the organisms and of their function, that may be associated with the activity of the bud mite pest, laying the foundations for further studies. Notably, this new piece of knowledge can be of great support to metatranscriptomics or metabolomics studies that may provide an in-depth understanding of the interactome between all the characters involved: hazelnut plant, the mite, and the associated microbiome. A wider scenario, that completes the outcomes of this study, would be provided by repeating the same investigation on more hazelnut varieties, to further assess eventual differences in the pool of organisms involved. The sampling time is an additional aspect that can be included in further experiments. It is widely known that, depending on the season and on the time of the year, there could be a variation of the different

species present in the buds and, accordingly, on big buds. Repeating the metagenomics analysis over time, covering all the seasons of the year, would extend the knowledge on the microbiome biodiversity and mite susceptibility, being of great support in the identification of key stages that maximise the effect of control strategies.

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CRediT authorship contribution statement

Angelo Mazzaglia: Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Stefano Speranza:** Supervision, Resources. **Mario Contarini:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Mounira Inas Drais:** Validation, Investigation. **Valerio Cristofori:** Supervision, Resources. **Cristian Silvestri:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Miloš Petrović:** Investigation, Formal analysis, Conceptualization. **Federico Brugneti:** Methodology, Investigation, Formal analysis, Conceptualization. **Irene Giubilei:** Validation, Investigation, Formal analysis. **Luca Rossini:** Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Silvia Turco:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127851.

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