



ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy Institute
of Biology Bucharest

PhD THESIS-SUMMARY

DYNAMICS AND APPLICATIVE POTENTIAL OF THE
MICROBIOME OF DIPTERAN SPECIES

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1. INTRODUCTION

Although lot of insects play important roles in ecosystems, many can be dangerous pathogens that can injure plants (Fliszkiewicz și Giejdasz, 2023); (Darwish, 2015).

Between plants and insects there are many types of relations, the latter being known as biological or mechanical vectors, with a great potential in food and plant contamination because of their variable food resources (Salerno et al., 2024; Dhokane și Chavan, 2020); (El-Sherbini, 2011); (Laroche et al., 2018).

Hemipterans can transfer pathogens to plants through mechanisms like ingestion or systemic spread, another reason for this type of spreading is the mutualism relations created with endosymbiotic bacteria (Heck, 2018).

Diptera can be very interesting from an scientific point of view, having multiple roles, like acceleration of the decomposing process or microorganism colonization from exoskeleton transported bacteria. Another source of fruit contamination can be represented by the insects regurgitation on fruit surfaces (Flury et al., 2018); (Park et al., 2017); (Vega et al., 2021); (Ercan et al., 2024).

Therefore the study of these species asociated with the dipteran from fruit orchards can help aquire vital information of their interactions and behavior and the consequences on the culture health and orchard production.

2. CURRENT STATE OF KNOWLEDGE

The uncontrolled proliferation of insects can be a cause for the loss of fruit trees, some of the species studied according this matter are *Drosophila melanogaster* (O'Grady și DeSalle, 2018), *Drosophila simulans* (Oakeshott et al., 1989); (Silva-Lopez et al., 2023) and *Drosophila*

subobscura (Merayo et al., 2025); (Mestres et al., 2004); (Marino et al., 2024). Other studied species and genera are *Botanophila fugax*, *Anthomyia* sp., *Scaptomyza pallida*, *Scaptomyza elmoi*, *Helina reversio*, *Delia platura*, *Neophyllomyza* sp., *Oscinella* sp., *Desmometopa sordida*, *Sapromyza* sp., *Musca domestica*, *Adrastus rachifer*, *Myzus cerasi*, *Fiebrigella* sp. and *Physiophora alceae*.

2.3. Ecological role of the insects

In Romania, *Ceratitis capitata* was studied and it was revealed that can infest a broad range of fruit trees (Thomas et al., 2001, rev. 2010); (Stanciu, 2007); (Chireceanu et al., 2013). A lot of local fruit orchards of plums, apples, cherries and peaches and here, the insects that can be found are *Drosophila*, *Musca*, *Delia* and *Anthomyia* and they are related with *Pseudomonas*, *Enterobacter* and *Acinetobacter* (Stoenescu et al., 2025); (Zhang et al., 2024; Li et al., 2025; Ranasinghe et al., 2023). Bacteria can be very important as a pathogen or as comensal species, by helping insects in various adaptive processes (Vallet-Gély et al., 2010; Wielkopolan et al., 2022); (Ranasinghe et al., 2023; Monyama et al., 2023).

2.4. Role of bacterial species associated with the dipteran species

For species as *Drosophila melanogaster*, it was demonstrated that exposure to bacterial communities may be of use for their growth, and also of an additional development of the reproductive organs was recorded when exposed to *Saccharomyce cerevisiae* or *Acetobacter malorum*. Whereas the absence of the exposure was associated with the decline in the reproductive processes (Qiao et al., 2019); (Elgart et al., 2016).

Another key factor in the organism development may be the diet that can influence the microbiome (Li et al., 2021); (Mogren & Shikano, 2021); or the microbiome can be enriched with entomopathogenic fungi and they can be employed as biocontrol agents (Picciotti et al., 2023).

Pathogen species present in the insect microbiome can be transmitted through various feeding strategies mediated by their mouthparts (Jiang et al., 2019); (Salerno et al., 2024); (Picciotti și al., 2023). Another pathogen transmission pathway may be represented by egg deposits within the fruits (Lizama et al., 2025); (Hazir et al., 2021).

Symbiotic relations are present between plants and insects and between insects and bacteria, having a very important role in pollination, digestion, feeding and reproduction (Salerno et al., 2024); (Holt et al., 2024).

3. PURPOSE AND OBJECTIVES OF THE THESIS

The aim of this doctoral thesis was to investigate the entomological and microbial diversity associated with fruit trees in the southern region of Romania (Clătești village, Călărași county) through an integrative approach based on genetic methods.

Specific objectives of the study

- (1) genetical identification based on DNA barcoding of insect species associated with various fruit trees that belongs to *Malus domestica*, (apple), *Malus domestica* "Jonagold" (Jonagold apple), *Prunus avium* (cherry), *Prunus persica* (peach), *Cydonia oblonga* (quince) species, and the assessment of their preferences for different pomological species.
- (2) new species report for Romanian entomofauna identified through genetic methods.
- (3) the bacterial communities associated with dipteran specimens belonging to the genera *Drosophila*, *Botanophila*, *Scaptomyza* and *Anthomyia*, collected from different types of fruit trees. The analysis of the taxonomical diversity of the bacterial communities was made through sequencing of the 16S rARN gene using Illumina. The evaluation of their distribution was made taking into account the host insect and the type of the fruit tree and the potential role of these bacteria in agent dissemination of the pathogens or the beneficial microorganisms from the orchard ecosystems.

4. MATERIALS AND METHODS

4.1 Study area and sample collection

The study area was a lowland region of southern Romania, in the Clatești village, Mitreni, Calarasi county. Specimens were collected using improvised traps that used as attractants bananas and vinegar (Bahrati et al., 2004), being afterwards stored in Eppendorf tubes and frozen.

4.2. Taxonomic identification of insects using morphological characteristics

Insect identification was performed by identifying morphological and genetic traits through DNA barcoding of the mitochondrial gene of the cytochrome c oxidase subunit I (COX1) (Hebert et al., 2003).

DNA extraction, PCR amplification

The extraction was conducted using the DNeasy Blood and Tissue Kit (Qiagen, Germania), that was followed by PCR amplification of the mitochondrial gene COX1 (Mioduchowska et al., 2018) using Mastercycler ProS System (Eppendorf, Germania). The resulting fragments were purified using the QIAquick PCR purification kit (Qiagen, Germania). The samples were concentrated at a minimum value of 2 ng/μL of DNA

Sanger sequencing of COX1 gene and the DNA sequencing analysis

The COX1 amplicons of the collected insects were sequenced by Macrogen (Netherlands), also, the nucleotidic sequences were edited using the Sequence Assembly and Alignment-CodonCode Aligner software (CodonCode Corporation, 2003) and they were identified by comparing them with homologous sequences from the NCBI GenBank database (Hebert et al., 2003), by applying an identity threshold of 97%. The sequences were deposited in the Genbank databases.

4.4. Characterization of insect-associated bacterial diversity through Illumina 16S rARN sequencing

Microbial DNA extraction, Illumina sequencing of the 16S rRNA gene, and sequence analysis

The total DNA extracted from the captured insects was isolated using the DNeasy Blood and Tissue kit (Qiagen, Germany) and was followed by the determination of the bacterial diversity associated with the insects by sequencing the region V3-V4 of the amplified gene 16S rRNA, and the amplicons were obtained and sequenced by Macrogen (South Korea) by using the platform Illumina MiSeq 300PE. The COX1 gene sequences were edited with the CodonCode Aligner software (CodonCode Corporation, 2003). The molecular identification was conducted by using the BLAST algorithm and the GenBank database (Altschul et al., 1990; Johnson, 2008). The taxonomical classification of amplicon sequence variants (ASVs) was carried out using the Silva v138 16rRNA database (silva.nr.v138) using the MicrobiomeAnalyst 2.0 platform (Lu et al., 2023).

Alpha and beta diversity indices were computed using the *phyloseq* package (McMurdie și Holmes, 2013). Differences in beta diversity were visualized through Non-Metric Multidimensional Scaling (NMDS) analysis (Kruskal, 1964). The statistical value of the observed clustering patterns was assessed using Analysis of Similarities (ANOSIM) (Clarke, 1993) and the Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) by taking into account an significance threshold of $p < 0,05$.

The statistical analysis were conducted using the platform MicrobiomeAnalyst 2.0 (Lu et al., 2023). Comparisons of the mean taxon abundances between sample groups were conducted using the Student's t-test (Wickham, 2016; Mishra et al., 2019; R Core Team, 2023).

RESULTS

5. INSECT SPECIES DISTRIBUTION ON FRUIT TREES IN AN ORCHARD AREA OF SOUTHERN ROMANIA

5.1 Insect sampling from fruit trees and habitat characterization

The fruit trees analyzed were *Malus pumila* (apple), *Malus domestica* Jonagold (Jonagold apple), *Prunus persica* (peach), *Prunus persica* platycarpa (flat peach), *Prunus avium* (cherry), *Cydonia oblonga* (quince) și *Prunus domestica* (plum). A total of 51 insect specimens were collected from these tree hosts, the majority originating from cherry trees. During the sampling period the mean temperatures were of approximately 24°C, and the trees had fruit in different developmental stage.

5.2 Morphologic and genetic identification of insects associated with fruit trees and analysis of their numerical and taxonomic variability

Taxonomic identification of collected insects was carried out based on morphological characteristics (Ackland, 2001; Komzáková și Rozkošný, 2009) revealing representatives of eight Diptera families (*Anthomyiidae*, *Drosophilidae*, *Milichiidae*, *Chloropidae*, *Muscidae*, *Aphididae*, *Ulidiidae* and *Lauxaniidae*), and of a specimen belonging to the *Elateridae* family (Coleoptera).

The COXI nucleotidic sequences of the insect specimens were identified through DNA barcoding and deposited in GenBank under accession numbers OK380902–OK380942, PV153587, PV017878 și PV018814–PV018823.

BLAST analysis (Zhang et al., 2000) of the COXI fragments revealed 18 different species of insects from Diptera order (*Botanophila fugax*, *Helina reversio*, *Sapromyza* sp., *Anthomyia* sp. ZFMK, *Anthomyia* sp., *Musca domestica*, *Drosophila melanogaster*, *D. subobscura*, *D. simulans*, *Neophyllomyza* sp., *Myzus cerasi*, *Delia platura*, *Oscinella* sp., *Scaptomyza pallida*, *S. elmoi*, *Fiebrigella* sp., *Desmometopa sordida* and *Physiophora alceae*).

From the total insect specimens, 24 were collected from cherry traps, 12 from apple traps, six from the peaches and four of each plum and quinces.

A Venn diagram showed the occurrence across the four types of fruit trees of the insects and indicated host-specific distribution patterns for most species, except for *Anthomyia* sp. and *Drosophila melanogaster* which were identified in more than one tree.

5.3. Effects of temperature and fruit phenology on insect dynamics

Of the 51 specimens collected, 24 were captured in July, 14 in June, nine in August and only two in May. Insect abundance was correlated with the fruiting stage, with cherries, that had ripe fruits, yielding the highest numbers, although 12 specimens were also collected from traps placed in trees bearing unripe fruits.

Species of *Drosophila* genus were consistently associated with peaches, while their presence varied among other tree species, showing a clear preference for ripe fruits.

5.4 Discussion

Insect dynamics in fruit trees from southern Romania

Previous studies in Romania have focused on forest ecosystems, this study additionally integrates molecular analyses, providing valuable insights into biodiversity assessment.

Role of temperature in the population dynamics of insects

Temperature exerts a strong influence on insect community dynamics, with higher abundance and diversity observed during warmer months.

Influence of the fruit phenology on the dynamics of insect population

The phenology of the fruit can significantly affect the insect preferences, and it is largely driven by sensory cues such as odor and color (Cunningham et al., 2016).

Dynamics of the entomofauna associated with the studied fruit trees and their ecological significance

Among the 19 insect species identified, 13 showed selective associations with specific fruit tree hosts, while only three species (*Anthomyia* sp., *Scaptomyza* sp. and *Drosophila melanogaster*) had been recorded across all fruit tree species. The *Anthomyia* individuals exhibited increased activity under moderate to high temperatures. *Drosophila* species, known for their adaptability, have been documented in southern Romania (Singh, 2015; Agustin et al., 2024; Coughlan et al., 2022) and in regions such as South Africa and Hawaii on a variety of exotic fruits (Oakeshott et al., 1989); (Ort et al., 2012).

6. EXPANSION OF THE DISTRIBUTION AREA OF THE REPORTED DIPTERAN SPECIES FOR THE FIRST TIME IN ROMANIA

6.1 *Botanophila fugax*

B. fugax is known for its diverse trophic relationships (Smith, 1989) and in this study were collected five specimens at moderate temperatures in July, subsequently being confirmed through genetic identification as the first records of this species in Romania. It exhibits distinct morphological characters, particularly in the reproductive structures (Ackland, 2001). The species has a medium distribution across Europe (GBIF) and the studies show that the species can be a potential vector in pathogen transmission or it can contribute to fungi reproduction cycle (Rossmann et al., 2018), (Pagel et al., 2019).

6.2 *Helina reversio* Harris 1780

Helina reversio has a holoartic distribution, having multiple food sources, also acting as an important pollinator (Pont, 2012); ([Commanster.eu](https://commanster.eu), 2025). In the present study, a single specimen was collected at the end of July and was identified both by morphological traits and DNA barcoding

6.3 *Scaptomyza elmoi* Takada 1970

Is a saprophagous insect, with ecological importance as a vector and as a food source for insectivorous plants or spiders (Stewart, 2021). The mains sources for food consist in decaying matter, and it can be found in areas with dense vegetation (Leblanc et al., 2009); (DigitalNZ, 2016). In this study, *S. elmoi* was collected in June from traps placed in cherry trees at moderate temperatures, and it was identified using both morphological and genetic approaches. The genus *Scaptomyza* has a wide distribution across Europe, while *S. elmoi* itself reported for the first time in Romania through this study (GBIF, 2025).

6.4 *Drosophila subobscura* Collin 1939

Drosophila subobscura is a cosmopolit species that can be present in colder areas like deciduous forests also depending on the decaying matter for food and reproduction (Krimbas și Loukas, 1980); (Smart, 1945). In the present study, two specimens were isolated, in June, based on morphological and genetic traits (COXI gene), representing the first records of this species in Romania

6.5 *Drosophila simulans* Sturtevant, 1919

Drosophila simulans is morphological similar to *Drosophila melanogaster*, being more sensitive to environmental conditions. It is a diurnal, cosmopolitan species with saprophagous feeding habits (David et al., 1982); (Chandler et al., 2011). In the present study, a single specimen was isolated, in July, and it has been identified by its distinctive reproductive structures as well as by genetic and morphological analyses. This represents the first report of *D. simulans* in Romania

6.6 *Fiebrigella* sp. Duda 1921

Species of the *Fiebrigella* genus can be found in pastures and meadows but also in decaying matter, on which they can feed upon. Adults may also consume nectar, sap or bee storage nutrients while larvae are mycophagous (Bodsworth et al., 2005); (Smith et al., 2008); (Hall și Gerhardt, 2002). In the present study were identified three specimens, collected in June, that were identified by morphological and genetical traits. This represents the first report of the genus *Fiebrigella* in Romania (GBIF, 2025).

6.7 Discussion

Within this doctoral study, the following species has been identified and reported for the first time in Romania: *Drosophila simulans*, *Drosophila subobscura*, *Fiebrigella* sp., *Scaptomyza elmoi*, *Botanophila fugax* and *Helina reversio* (Global Biodiversity Information Facility); (Copoiu și Purcărea, 2023; Copoiu et al., 2025). These findings represent significant contributions to the understanding of insect distribution in the Southern European region. *Helina reversio* and *Scaptomyza elmoi* show a wide distribution that ranges across Europe, while *Drosophila subobscura* and *Drosophila simulans* are more frequent in northern and western parts of the continent. The genus *Fiebrigella* has been recorded in only a few European countries, with no direct geographical continuity among them, and *D. simulans* was not reported previously in any of the Romanian neighboring countries.

This first regional record of six dipteran species within fruit tree agroecosystems in southern Romania provides a valuable update on the entomofaunal distribution in Europe and opens new research directions regarding their ecology, trophic behavior and potential agronomic roles, whether beneficial or detrimental.

7. ASSESSMENT OF BACTERIAL COMMUNITIES IN INSECTS ASSOCIATED WITH FRUIT TREES FROM THE CLĂTEȘTI AREA, ROMANIA

7.1 Bacterial diversity associated with insects inhabiting fruit trees

The characterization of the diversity of bacterial communities associated with the collected insects and the assessment of their variability according to the host fruit tree species were carried out using Illumina sequencing of the 16S rRNA gene. The analysis included 19 specimens belonging to the genera *Botanophila*, *Anthomyia*, *Drosophila* and *Scaptomyza* each

being represented by a minimum of two samples, selected from the total of 51 species collected from apples, plums, cherries, peaches and quinces. The microbiome of the *Drosophila* and *Anthomyia* exhibited the greatest variability in bacterial load, with some specimens harboring over 1,500 ASVs, having a similar pattern as the genus *Botanophila*. In contrast, the *Scaptomyza* genus presented a lower diversity with lower ASVs.

Alpha diversity

Serves as an indicator of the structure of insect-associated microbiomes, reflecting both taxonomic richness and the evenness of distribution (Willis, 2019). Sample AI1 exhibited the highest diversity, corresponding to Shannon (7.02), Fisher (328.8) and Chao-1 (1595) with a high evenness index of 0.7016. In contrast, sample PR2 showed the lowest values for the same indices Shannon (4.125), Fisher (15.12), Chao-1 (138) reflecting a reduced diversity and a mixed population lacking apparent equilibrium, as indicated by the evenness value of 0.4482. The G5 sample showed the least uniformity (0.2735), suggesting a dominance of certain species corroborated by moderate Shannon and Chao-1 indices.

These results highlight a substantial variability in the structure of bacterial communities associated with different insect species. The microbiome of sample AI1 had been distinguished by high bacterial diversity and evenness, whereas PR2 and G5 reflected reduced diversity and low dominance of particular taxa.

Principal Coordinates Analysis (PCoA) based on Chao-1 values indicated significant variation in bacterial diversity according to the type of fruit tree. Insects associated with *Malus domestica* and *Prunus avium*, while insects collected from *Cydonia oblonga* showed the lowest bacterial diversity, as all specimens from this host were *Drosophila melanogaster*. The observed distributions and elevated median values suggest that, despite taxonomic similarity, species within the same genus can host bacterial communities with substantial variability.

At genus level, *Drosophila* exhibited high variability in bacterial diversity, whereas *Botanophila* and *Anthomyia* presented moderate diversity according Chao-1 indices. *Scaptomyza* displayed a less diverse bacterial community. These inter-genus differences may be influenced by feeding behaviors as well as interactions with host plants (Naveed et al., 2024).

Beta diversity

Non-metric multidimensional scaling (NMDS) of ASVs revealed that the distribution of insect-associated bacterial communities varied according both the host fruit tree species and the insect genus. Bacterial communities exhibited significant variation based on the host tree

species (ANOSIM - $R = 0,79$; $p < 0,001$), indicating a clear and statistically significant separation between groups, with minimal overlap. The high R-value suggests marked differences in bacterial community structure according to the host tree species. The formation of distinct clusters, as well as partial overlaps, indicates that species-specific factors of the fruit trees play a key role in shaping the structure of insect-associated bacterial communities. In contrast, bacterial community composition did not show significant differences according to the insect genus.

7.2 Taxonomic profile of insect-associated bacterial communities

The 16S rRNA amplicon sequence variants (ASVs) identified in the studied insects were taxonomically classified into four phyla, six classes, 17 orders, 31 families and 51 bacterial genera, reflecting a complex and well-structured microbial diversity. Taxa from the phyla *Pseudomonadota* and *Bacillota* were relatively dominant across all insect samples.

Pseudomonadota dominated bacterial communities in insects collected from *Malus domestica* Jonagold, *Prunus domestica* and *Prunus persica* whereas *Bacillota* showed relatively high abundance in samples from *M. domestica* and *Prunus avium* sharing a core taxonomy with *Pseudomonadota*. The phylum *Actinomycetota* was found at low abundance in insects from *Cydonia oblonga*, with limited presence in samples AI1 and N1 from Jonagold apple and peach. *Bacteroidota* was identified only in sample AI1 from Jonagold apple, indicating a restricted distribution within the analyzed Diptera.

At class level, bacterial communities were dominated by *Pseudomonadota* and *Bacillota* phyla, while at genus level, substantial variability was observed among bacterial communities associated with insects from different fruit tree species. Among the identified taxa, the *Gluconobacter* genus was prevalent in insects from all fruit tree types, whereas *Wolbachia* showed high abundance in PR2 and AI2 microbiomes but was completely absent in insects from *Cydonia oblonga* and *Prunus persica*. Notably, *Fructobacillus* species were more frequent in *Prunus avium* and *Malus domestica*, while *Acetobacter* displayed a more uniform distribution, being abundant in *Malus domestica* JONAGOLD, but less so in *Malus domestica*.

The Heatmap analysis of bacterial community density across the four insect genera (*Anthomyia*, *Botanophila*, *Drosophila* and *Scaptomyza*) showed significant difference in both relative abundance and composition. The microbiome of *Drosophila* was characterized by a

high prevalence of *Bacillus*, *Morganella* and *Pantoea*. *Scaptomyza* exhibited a distinct microbial profile dominated by *Pseudomonas*, *Corynebacterium* and *Staphylococcus*, *Anthomyia* specimens presented homogeneous bacterial diversity, while *Botanophila* insects were marked by elevated relative abundance of *Fructobacillus*, *Wolbachia* and *Lactobacillus*.

Among the bacterial genera identified, *Klebsiella*, *Enterobacter*, *Bacillus*, *Acinetobacter*, *Staphylococcus*, *Corynebacterium*, *Enterococcus* and *Bacteroides* all being known for being potential pathogens for humans and were detected across various insect samples with variable abundance levels.

7.3 Discussion

Dynamics of bacteria-insect-fruit tree interactions and the potential role of dipterans as transmission vectors

The analysis of taxonomic diversity according to the host fruit tree species and insect genus (*Anthomyia*, *Botanophila*, *Drosophila* and *Scaptomyza*) in specimens collected from fruit trees in the Clătești region, Călărași county revealed variability dependent on these two factors.

The bacterial communities associated with these dipterans belonged to the phyla Proteobacteria, Firmicutes, Bacteroidota and Actinobacteria, with a clear dominance of taxa from the phylum Proteobacteria.

The taxonomical variable diversity among the four insect genera indicated the highest total number of bacterial phyla in *Drosophila* (four phyla) being followed by *Anthomyia* and *Botanophila* (three phyla each), whereas *Scaptomyza* harbored bacteria from only two phyla. These results suggest a differentiated microbial diversity among insect genera, influenced by their ecology and potential trophic or habitat related interactions.

The analysis of the microbiome of the insects collected from different fruit trees highlighted a significant variability in bacterial composition depending on the host.

Several of the analyzed insects also carried bacterial species with potential pathogenicity, as well as species with beneficial roles, both of which could be transmitted to the insect hosts or to their individuals within the same species

8. GENERAL CONCLUSIONS AND PERSPECTIVES

This doctoral study represents the first complex characterisation of the relationships between fruit trees species, associated insects and bacterial communities from Romania,

generating original data that contributes to a better understanding of microbial and entomological ecology within local agroecosystems and providing a foundation for future studies on the role of insects in the dissemination of microorganisms among plants.

Within the study conducted in southern Romania in Clătești, Călărași county, during May-August 2021, a total of 51 insect specimens were collected from various fruit tree species, including *Malus pumila*, *Malus domestica* 'Jonagold', *Prunus domestica*, *Prunus avium*, *Prunus persica*, *Prunus persica* var. *platicarpa* and *Cydonia oblonga*.

Insect species were identified through DNA barcoding using mitochondrial COXI gene sequencing, while the bacterial communities associated with dipterans of the genera *Anthomyia*, *Botanophila*, *Drosophila* and *Scaptomyza* collected were characterized via Illumina sequencing of the 16S rRNA gene.

Insect dynamics in fruit trees

Most of the isolated species belonged to the *Drosophila* genus across all types of fruit trees, whereas most of the other insect genera were represented by a single specimen. Species of the genus *Anthomyia* were present on apple and plum trees, *Scaptomyza* species were found on cherry and peach trees, and *Botanophila fugax* specimens were collected from apple trees.

The highest insect diversity was recorded on cherry and apple trees, with fruit ripeness being a key factor. *Anthomyia* sp. and *Scaptomyza* sp., were common to two fruit tree types, while *Drosophila melanogaster* was found across all fruit tree species.

Dipteran species newly reported in Romania

Specimens of Diptera belonging to the species *Fiebrigella* sp. [OK380918], [OK380920] and [OK380922]; *Drosophila simulans* [OK380938], *Drosophila subobscura* [OK380908] and [OK380906]; *Helina reversio* [OK380925], *Scaptomyza elmoi* [OK380933], *Botanophila fugax* [OK380924], [OK380928], [OK380929], [OK380932] and [PV018814] had been reported for the first time in Romania in this study following genetic identification based on the COXI gene, thereby extending the known distribution range of these Diptera across Europe.

Diversity and potential role of microbial communities associated with orchard insects

Analysis of the bacterial microbiota revealed significant variations among the Diptera genera investigated, both in terms of phylogenetic diversity and distribution across bacterial

phyla. Genera *Anthomyia* and *Botanophila* were associated with bacteria from Proteobacteria, Firmicutes and Bacteroidota, whereas *Scaptomyza* was associated only with bacteria from Proteobacteria and Firmicutes. *Drosophila* genus had presented the most extended bacterial diversity with the present phyla: Proteobacteria, Firmicutes, Bacteroidota and Actinobacteria.

All studied genera were dominated by bacteria from Proteobacteria phyla, and *Anthomyia*, *Botanophila* and *Scaptomyza* had frequently presented the Firmicutes phyla. Actinobacteria phyla was exclusively identified at *Drosophila*, which may reflect enhanced adaptive capabilities relative to available food sources.

Phylum *Bacillota* was predominant in insects collected from apple and cherry, while *Pseudomonadota* was associated with plums, peaches and Jonagold apples.

Alpha diversity analysis of the ASVs obtained via Illumina sequencing of the 16S rRNA gene from the insects microbiomes demonstrated high variability of bacterial communities across fruit tree species, indicating species-specific influences. NMDS analysis of beta diversity further highlighted pronounced differences in the insect microbiomes according to the host tree species.

In contrast, variations on microbial composition among insect genera were not statistically significant, except for the genus *Drosophila* which showed considerably higher bacterial diversity compared to the other analyzed genera.

These results support the hypothesis that the identity of the plant host constitutes a major determinant in structuring the bacterial communities of insects, exerting a stronger influence than the phylogeny of the entomological host.

The variability of bacterial composition at the genus level, identified among the Diptera, highlighted the presence of potentially pathogenic bacteria. At the same time, certain bacterial genera identified within the microbiomes of insects collected from fruit trees may confer functional benefits to their hosts, contributing to processes such as digestion, synthesis of essential compounds and protection against pathogens.

The remarkable diversity of the microbiome in insect species belonging to the genera *Anthomyia*, *Botanophila*, *Drosophila* and *Scaptomyza*, collected from the five fruit tree species in southern Romania, suggests that these Diptera may play an important role in facilitating microbial transfer among apple, cherry, peach, plum and quince trees, indicating the existence of a multifactorial mechanism of microbial dispersal influenced both by substrate characteristics and various environmental factors

Perspectives

For a deeper understanding of the relationship between insects and their microbiomes in the context of fruit trees, future research should aim to expand the geographical range of sampling and increase the number of specimens analyzed, allowing for a more robust statistical characterization of bacterial diversity.

Furthermore, incorporating sex differentiation in the collection and analysis process could reveal potential variations in the microbiome according to the reproductive physiology of the insects, providing a more detailed perspective on sex-specific microbial ecology.

Another important approach would be to extend the temporal scope of the study to cover the entire growing season- from flowering to full fruit ripening- to capture potential seasonal variations in the microbiome and the dynamics of insect distribution in relation to host plant development.

In addition, experiments conducted in controlled environment, where insects are reared and fed exclusively on plant material from a single fruit tree species, would allow the direct evaluation of dietary influence on microbiome composition, excluding interference from other trophic sources.

These research directions could contribute significantly to understanding the mechanisms of interaction among insects, plants and their microbiomes, with potential applications in plant protection, biological control, and the management of phytopathogenic diseases.

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