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BRIEF REPORT



## Monophagous olive moth and its bacterial microbiota: unveiling the holobiont towards pest management

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### ABSTRACT

Symbiotic-based pest management strategies are promising but require previous knowledge on the microbial community structure and on key microorganisms. Highly specialised pests, as the monophagous olive moth, are likely to have co-evolved microbiota that aid in overcoming specific plant defences that have emerged from the tight insect-plant interaction. Here we report a first attempt to identify olive moth bacterial microbiome based on 16S sequencing. This preliminary analysis intended to highlight potential host-symbiont interactions, representing key aspects in the moth survival and adaptation, such as nutrition (e.g. *Pseudomonas* sp.), detoxification (e.g. *Acetivobacter* sp. or *Staphylococcus* sp.), and protection/defence (e.g. Actinobacteria).

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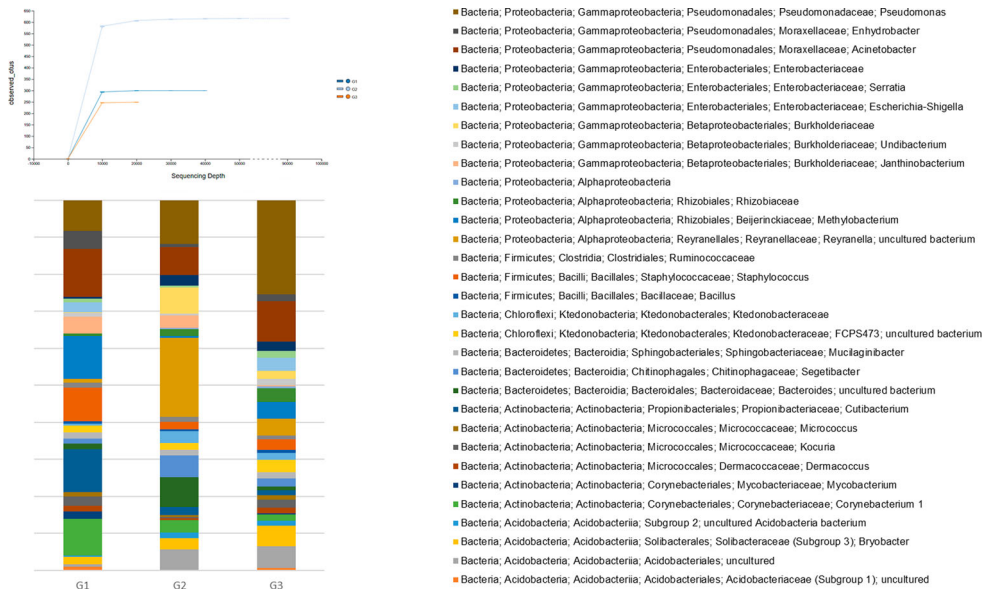
Natural enemies of pest are traditionally seen as parasites, pathogens, and predators that have the capacity of managing pests and their damage. However, this view can be enlarged to encompass the pest associated microbiota, as these microorganisms cannot be classified exclusively as beneficial, harmless or deleterious because their impact on the insect pest can depend on circumstance. Exploring this (in)balance is one way that a symbiotic-based pest management strategy can develop. A pre-requisite for such an approach is the knowledge of the microbiota associated with the insect pest and its determinants.

The olive moth, *Prays oleae* (Bernard, 1978) (Lepidoptera, Praydidae) remains an abundant insect pest of olive trees of which not many natural enemies are known or exploited. It is a monophagous species with three annual generations, each one developing at a different organ from its olive host (*Olea europaea* L.): leaves (phyllophagous generation), flowers (antophagous generation) and fruits (carpophagous generation). Depending on the olive as only host for survival and reproduction, the olive moth had to evolve to counterpart the wide variety of secondary metabolites that the plant produces as a strategy of chemical defence to tolerate biotic (and abiotic) stresses. Amongst them, the olive contains several unusual secoiridoids commonly known as oleosides that are unique to Oleaceae, which the most significant is the oleuropein. This compound has been detected in various organs of the olive tree, like leaves, flowers, bark, and roots,

but it is more known from the fruit, being responsible for its bitter taste. Other phenolics compounds, like rutin, tannin, caffeic, verbascoside and luteolin are suggested to be involved in the defence against *Prays oleae* (Ilias et al., 2017). To be able to use olive nutrients, herbivores and microbes need to deal with abundant secondary metabolites. For example, the olive fruit fly -*Bactrocera oleae* (Rossi, 1790) (Diptera, Tephritidae)- evolved to harbour a vertically transmitted and obligate bacterial symbiont, the *Candidatus* *Erwinia dacicola*, that allows the insect to cope with the abundant secondary metabolites and in particular, with the oleuropein. It is unknown how the olive moth overcome the plant host defences. It is now widely accepted that holobionts -host organism and its symbiotic microbiota- are widespread in nature, making these entities the likely unit of selection and evolution. Hence the importance of addressing the olive moth and its microbiota as a whole: the selection pressure to overcome plant defensive compounds is exerted not only on the insect but also on the associated core bacteria. Here we report a first attempt to identify *Prays oleae* microbiome based on 16S sequencing, in search for potential symbionts that would aid in detoxification considering the plants defensive compounds. This knowledge is the first step towards identification and selection of symbionts that can be further used as a potential tool for population control.

Olive fruits were collected in September 2019, in Crete, Greece in a single olive orchard and the olives were brought into the laboratory and left in vials at room temperature until adult emergence (corresponding to the carpophagous generation). Individuals were stored at  $-20^{\circ}\text{C}$  in 70% ethanol until DNA extraction. Extraction was done per individual using the DNA Zymobiomics kit (Zymo Research<sup>®</sup>) and following the advised protocol. The NGS sequencing procedures, for the three insects independently, were performed at STABVIDA, Lda. (Portugal). After quality control of the DNA, to ensure samples had sufficient integrity and quantity for optimal amplification, the library construction was performed using the Illumina 16S Metagenomic Sequencing Library preparation protocol and the generated DNA fragments (DNA libraries) were sequenced with MiSeq Reagent Kit v3 in the Illumina MiSeq platform, for the V3 and V4 regions of the 16S rRNA gene, using 300 bp paired-end sequencing reads (available at NCBI, BioProject ID PRJNA640599). The analysis of the generated raw sequence data was carried out using QIIME2 v2019.10. Data denoising was done using the DADA2 plugin by trimming and truncating low quality regions, dereplicating the reads and filtering chimeras. The scikit-learn classifier was used to train the classifier using the SILVA (release 132 QIIME) database, with a clustering threshold of 97% similarity. For classification purposes, only OTUs containing at least 10 sequence reads were considered as significant.

The samples generated from 117,020–259,912 raw sequence reads, which is in accordance with the expected output. After denoising, a total of 1,017 unique features (OTUs) of 221 bp could be detected (with a total frequency of 170,771 in the three samples, being a sample defined by a single adult of olive moth). Even though the total amount of OTUs observed per sample varied considerably (also initial DNA concentration and integrity was different between samples, which might have led to this output differences), the alpha-rarefaction curve clearly reached a plateau indicating that the sequencing was deep enough to detect present 16S diversity (Figure 1). Almost all reads (99.35%) were associated with Bacteria.



**Figure 1.** Observed OTUs Alpha rarefaction curve and taxonomic identification of the shared OTUs per *Prays oleae* sample.

Defining, conservatively, the core microbiota as the bacteria present in all 3 samples with more than 10 reads per sample, 31 classified groups emerged (Figure 1). A high number of reads correspond to non-classified bacteria -the most frequent OTU (11,056) was classified to the Bacteria level only using the SILVA (with a 98% similarity), as well as 16 others shared OTUs. The most frequent bacteria was classified as *Pseudomonas* sp., a ubiquitous genus with high metabolic diversity, often encountered associated to insects as an entomopathogen, but also showing a high metabolic versatility with amylolytic, cellulolytic, xylanolytic, lipolytic, and esterase activities (Jing et al., 2020). Also, there are references to *Pseudomonas* sp. aiding in detoxification processes including degradation of insecticides. Still within the Pseudomonadales, but belonging to Moraxellaceae, the presence of *Acinetobacter* sp. (5949 reads) and *Enhydrobacter* sp. (1442 reads) should be referred. *Acinetobacter* are commonly found in several systems and are reported by their capacity to degrade large molecules, including pesticides (Geiger et al., 2009). *Enhydrobacter*, like *Pseudomonas* and *Acinetobacter*, has also been assigned to the core microbiota of other insect pest moths.

*Pseudomonas*, together with *Bacillus*, *Staphylococcus*, *Enterobacter*, and *Enterococcus*, are the most widespread bacterial genus in lepidopteran species (Voiron et al., 2018). Surprising enough, no OTU was classified as *Bacillus*, and the main Firmicutes found in the three samples was a *Staphylococcus* (2767 reads in total, considering 9 OTUs with only 10 polymorphic sites). *Staphylococcus* sp. was found to provide resistance to specific toxic compounds - alkaloids and latex- in two lepidopterans (Vilanova et al., 2016). Nonetheless, they are widespread in insects and have been traditionally considered generalist bacteria, highly prevalent in the Lepidoptera families Sphingidae and Noctuidae (Visôto et al., 2009). As to the Enterobacteriaceae, two OTUs could be found in all samples: one classified as *Escherichia-Shigella* (1008 reads) and a *Serratia* sp. (543 reads). Other

Enterobacteriaceae were found comprising 9 OTUs (2754 reads in total). Species of the genus *Serratia* are known to be associated with insects as pathogens (Inglis & Lawrence, 2001) and as symbionts (Latorre et al., 2012), or its presence in insect's digestive tract can just be of plant/environmental origin.

Two Alphaproteobacteria are predominantly present in the three samples: *Reyranella* sp. (6493 reads) and *Methylobacterium* sp. (3054 reads). The first is associated with different soils, including agricultural and can thus associate to the insect's digestive tract (albeit establishing during oviposition and in olive development, as the adults were captured upon eclosing in laboratory conditions). The second, a methane-utilizing bacterium, has ubiquitous occurrence on plant leaves and has been detected on table olives (Cocolin et al., 2013).

The Actinobacteria are known for protective insect-actinobacteria associations, and symbiotic relations might be more common than previously thought. In *Prays oleae*, five actinobacteria species were present in all samples: in the Propionibacteriales, *Cutibacterium* sp. (3142 reads); in the Corynebacteriales, *Corynebacterium* sp. (3126 reads) and *Mycobacterium* sp. (479 reads); and in the Micrococcales, *Kocuria* sp. (884 reads), *Dermacoccus* sp. (599 reads), *Micrococcus* sp. (475 reads). Also deserving reference is the presence of a Bacteroides in all samples (2570 reads).

This preliminary analysis of the olive moth bacterial microbiota intended to highlight potential host-symbiont interactions, representing key aspects in the moth survival and adaptation, such as nutrition (e.g. *Pseudomonas* sp.), detoxification (e.g. *Acetivibrio* sp. or *Staphylococcus* sp.), and protection/defence (e.g. Actinobacteria). A comprehensive study is now needed, including looking for a conserved microbiota across the different developmental stages of *Prays oleae* of the three generations developing on the olive tree followed by a functional profiling of the core microbiome. This is a necessary condition for the delineation of a potential pest management strategy based on symbiosis. Arora and Douglas (2017) provided a critical review of multiple pest management approaches available targeting the insect as an holobiont, founded on manipulation of microbial partners. These approaches have been gaining recognition over time, becoming more relevant in the context of modern sustainable agriculture.

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## Data availability statement

The data that support the findings of this study are openly available in NCBI with the code Bio-Project ID PRJNA640599.

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