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The effects of geographic origin and antibiotic treatment on the gut symbiotic communities of *Bactrocera oleae* populations

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Abstract

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the major insect pest of olive orchards (*Olea europaea* L.), causing extensive damages on cultivated olive crops worldwide. Due to its economic importance, it has been the target species for a variety of population control approaches including the sterile insect technique (SIT). However, the inefficiency of the current mass-rearing techniques impedes the successful application of area-wide integrated pest management programs with an SIT component. It has been shown that insect mass rearing and quality of sterile insects can be improved by the manipulation of the insect gut microbiota and probiotic applications. In order to exploit the gut bacteria, it is important to investigate the structure of the gut microbial community. In the current study, we characterized the gut bacterial profile of two wild olive fruit fly populations introduced in laboratory conditions using next generation sequencing of two regions of the 16S *rRNA* gene. We compared the microbiota profiles regarding the geographic origin of the samples. Additionally, we investigated potential changes in the gut bacteria community before and after the first exposure of the wild adult flies to artificial adult diet with and without antibiotics. Various genera – such as *Erwinia*, *Providencia*, *Enterobacter*, and *Klebsiella* – were detected for the first time in *B. oleae*. The most dominant species was *Candidatus Erwinia dacicola* Capuzzo et al. and it was not affected by the antibiotics in the artificial adult diet used in the first generation of laboratory rearing. Geographic origin affected the overall structure of the gut community of the olive fruit fly, but antibiotic treatment in the first generation did not significantly alter the gut microbiota community.

Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), lays its eggs in the mesocarp of the olive fruit. The occurring specialist's larvae develop only on olive fruits, causing damage to the quality of both table olives

and the produced olive oil (Levinson & Levinson, 1984; Manousis & Moore, 1987). Current strategies against *B. oleae* are mostly based on mass trapping, bait sprays, and insecticides (Haniotakis, 2005). However, bait sprays and insecticides have drawbacks, such as the emergence of insecticide resistance and a negative impact on non-target species as well as on human health (Haniotakis, 2005; Daane & Johnson, 2010; Kakani et al., 2010). The negative side effects of these methods emphasize the current need for the development of integrated pest management

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(IPM) strategies that combine environmentally friendly population control methods in an area-wide approach (Klassen, 2005; Hendrichs et al., 2007).

A method used as a component for the IPM of a variety of fruit pests is the sterile insect technique (SIT), based on mass production and release of irradiated sterile males that compete with wild males for mating with wild females. Irradiation causes chromosomal breaks which lead to males that carry lethal mutations in their sperm. The offspring of the irradiated males that mate with wild females are not viable, subsequently leading to population decline (Knippling, 1955; Dyck et al., 2005). One of the most crucial steps in large-scale applications, such as the SIT strategy, is the efficient mass rearing of robust flies with high survival in the field and high competitiveness with their wild counterparts. An essential prerequisite for the development of such flies is the understanding of insect biology, which was investigated in a number of older studies from 1950s to 1980s (Economopoulos, 1972, 1977; Economopoulos et al., 1976; Economopoulos & Loukas, 1986). However, the extremely laborious and cost-intensive mass-rearing procedure impeded any further progress (Estes et al., 2011). One of the challenges faced in the mass rearing of *B. oleae* is the development of an adequate artificial larval diet, due to the monophagy of the larvae (Manoukas, 1975; Estes et al., 2011; Ras et al., 2017), a common issue with specialist feeders (Parker, 2005). Although current artificial diets allow larval development, they are still expensive, laborious, and do not provide consistent survival or quality of all life stages of the insect (Estes et al., 2011; Ahmad et al., 2014; Ras et al., 2017). It is thus important to find alternative and/or supplementary ingredients to improve the nutrition quality of larval diets.

Insect symbiosis plays an important role in a wide variety of life-history traits (Bourtzis & Miller, 2003, 2006, 2008; Vega & Dowd, 2005; Zchori-Fein & Bourtzis, 2011; Engel & Moran, 2013; Kyritsis et al., 2017), providing benefits to their hosts that contribute to their fitness enhancement and general health. In particular, microbes residing in the insect digestive system are directly associated with insect nutrition. Several studies in *Ceratitis capitata* (Wiedemann), the tephritid model for mass rearing and SIT applications, demonstrated that gut symbionts as supplements in the adult or larval diet have positive effects on a variety of life-history traits related to SIT applications (Niyazi et al., 2004; Behar et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Augustinos et al., 2015; Kyritsis et al., 2017).

Several older studies focused on the interaction of the olive fruit fly with gut microbiota using mostly cultivation-dependent methods that characterize bacteria able to grow in selective media (Petri, 1909; Hellmuth, 1956;

Yamvrias et al., 1970; Tzanakakis & Stavrinides, 1973; Lüthy et al., 1983; Tsiropoulos, 1983; Manousis & Ellar, 1988; Stamopoulos & Tzanetakis, 1988). More recent cultivation-independent methods that involve analysis of the 16S *rRNA* gene using conventional molecular methods or next generation sequencing (NGS) approaches, identified previously undetected bacteria (Capuzzo et al., 2005; Sacchetti et al., 2008; Estes, 2009; Estes et al., 2009; Ben-Yosef et al., 2010). The major symbiont identified in these studies was *Candidatus* *Erwinia dacicola* Capuzzo et al., a Gammaproteobacterium of the Enterobacteriaceae family, which so far cannot be cultivated in any bacterial medium. It exists in both intra- and extracellular form (Capuzzo et al., 2005; Estes et al., 2009) and plays an important role in facilitating the development of the olive fruit fly larvae in the hostile phenolic environment of unripe olives (Ben-Yosef et al., 2015) and in the enhancement of nitrogen fixation in adult flies (Ben-Yosef et al., 2014). *Candidatus* *E. dacicola* is the predominant species detected in wild *B. oleae* populations from several geographic regions (Capuzzo et al., 2005; Sacchetti et al., 2008; Estes et al., 2009, 2012; Kounatidis et al., 2009; Savio et al., 2011). *Providencia* sp. (Estes et al., 2014), *Enterobacter* sp. (Stamopoulos & Tzanetakis, 1988; Estes, 2009), *Acetobacter tropicalis* (Lisdiyanti et al., 2000; Kounatidis et al., 2009), *Pantoea* sp. (Ben-Yosef et al., 2015), *Klebsiella* sp., and *Serratia* sp. (Tsiropoulos, 1983; Konstantopoulou et al., 2005) have also been identified in lower densities in wild populations (reviewed in Ras et al., 2017). Adaptation to laboratory conditions leads to the loss of *Ca. E. dacicola*, a decrease in bacterial diversity (Tsiropoulos, 1983; Konstantopoulou et al., 2005; Kounatidis et al., 2009; Estes et al., 2012; Ben-Yosef et al., 2015) and colonization by other potentially pathogenic species such as *Morganella morganii* (Winslow et al.) Fulton (Estes, 2009; Estes et al., 2011; AA Augustinos, G Tsiamis, C Caceres, AMM Abd-Alla & K Bourtzis, in preparation).

Several recent trials to improve artificial rearing indicated the difficulties in laboratory domestication of *B. oleae* (Ahmad et al., 2014, 2016; Zygouridis et al., 2014). For instance, Zygouridis et al. (2014) demonstrated a dramatic reduction (98%) of the original population in the initial generations (F0–F2) during laboratory adaptation. Natural differences in the gut microbiota composition could be the reason for these population drops and often colony collapses in many laboratories, due to the replacement of olives with artificial larval diet. However, the exact causes have not been fully addressed yet. In the current study, we performed Illumina NGS of two 16S *rRNA* gene regions in samples of two wild *B. oleae* populations before their introduction to laboratory conditions and in the first generation after the introduction.

Our aim was to study the effect of laboratory rearing and antibiotic treatment on the gut microbiota composition of the olive fruit fly.

Materials and methods

Bactrocera oleae populations and rearing conditions

Wild *B. oleae* were collected from infested olives in orchards from two regions in Greece. Population 1 (P1) came from pupae collected in November 2014 in Volos, population 2 (P2) came from larvae collected in January 2016 in Crete. The larvae from P2 were collected directly from olives and were not fed with any artificial diet. The flies that emerged from both populations were fed with standard laboratory adult diet consisting of 75% sugar, 19% hydrolyzed yeast, and 6% egg yolk powder without antibiotics. At the same time, flies that emerged from P1 were fed with laboratory adult diet containing 0.08% (dry weight) antibiotic (streptomycin), creating population 1A (P1A). For years, the addition of antibiotic was common practice (Tsitsipis & Kontos, 1983) that was considered to suppress potentially pathogenic bacteria that could decrease fecundity. However, recent studies demonstrated that antibiotic application in adult diet actually suppresses female fecundity (Dimou et al., 2010). Therefore, we decided to assess the addition of antibiotic in P1 but not in P2 due to the inadequate number of emerged flies. Insects of all life stages were cultured under constant environmental conditions at 25 ± 1 °C, $60 \pm 5\%$ r.h., and L14:D10 photoperiod.

Dissections and gut collections

Flies were immobilized at 4 °C and surface sterilized by washing in 70% ethanol and sterile phosphate-buffered saline. Guts were collected from third instars (P2 only), 1-day-old male and female adults (unfed) from P1 and P2, and 5-day- and 15-day-old males and females from P1, P1A, and P2. In addition, whole pupae were collected from P1 only. Three replicates per treatment were collected and each sample consisted of guts from five individuals. Samples were stored at -20 °C until DNA extraction. Detailed sample descriptions are given in Table S1.

DNA extraction and 16S rRNA gene amplicon library preparation and sequencing

Frozen guts were homogenized in liquid nitrogen using polypropylene pestles. DNeasy Blood and Tissue Kit (Qiagen, Vienna, Austria) was used for DNA extraction according to manufacturer's instructions. Samples were tested for DNA quality and quantity using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Vienna,

Austria) and diluted to a final concentration of $5\text{--}30$ ng μl^{-1} . PCRs and library preparation were performed by Macrogen and sequencing was performed using the Illumina MiSeq platform (Macrogen, Seoul, Korea). The primers 3F: AGAGTTTGATCMTGGC, 529R: ACCG CGGCKGCTGGC, 909F: ACTCAAAGGAATWGACGG, and 1391R:GACGGGCGGTGWGTRCA were used to amplify regions V1–V3 (3–529) and V6–V8 (909–1391) of 16S rRNA gene, respectively (Sogin et al., 2006; Cole et al., 2007; Eid et al., 2009; Klindworth et al., 2013).

Bioinformatics and statistical analysis

Raw sequence reads were assembled with usearch (v.10; Edgar, 2010) with the fastq_mergepairs command and filtered using the -fastq_filter option. Unique sequences were identified with the -fastx_uniques command and then clustered in operational taxonomic units (OTUs) with the -cluster_otus command. Sequences shorter than 200 bp were excluded. OTU tables were created with the -cluster_otus option. OTUs with a relative abundance below 0.005 were excluded from the analysis using the -otu_trim command. Chimeras were removed with the -unnoise3 option (Edgar, 2016). Taxonomy was assigned against SILVA (Quast et al., 2013) database using Qiime2 (Caporaso et al., 2010) and the BLAST algorithm (Altschul et al., 1990).

For quantifying alpha-diversity, the intrasample variation was calculated. Richness provided the value of OTUs present within one sample whereas the effective diversity of a microbial profile for a certain index is the number of equally abundant species that would give the same value for that index. Alpha-diversity was determined with the Rhea scripts as described previously (Lagkouvardos et al., 2017). Beta-diversity was calculated with generalized Uni-Frac (Chen et al., 2012). Visualization of the multidimensional distance matrix in a space of two dimensions was performed by the robust nonmetric version of multidimensional scaling (Minchin, 1987). A permutational multivariate ANOVA using distance matrices (vegan::adonis) was performed to determine whether the separation of groups was significant, as a whole and in pairs (Anderson, 2001). 16S rRNA gene sequences reported in this study have been deposited in NCBI under Bioproject PRJNA490261.

Results

16S rRNA-based taxonomic composition of the olive fruit fly populations

In total, 51 samples were analyzed and 1 213 564 reads were used for the bioinformatics analysis with an average of 23 795 reads per sample (Table S2). Twenty-one OTUs

Table 1 16S rRNA-based taxonomic composition of the gut bacteria of the three olive fruit fly populations 1 (P1), 1A (P1A), and 2 (P2)

Phylum	Class	Order	Family	Genus	P1	P1A	P2
Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	×	×	×
Deinococcus–Thermus	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>	×	×	
		Thermales	Thermaceae	<i>Meiothermus</i>			×
Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Geobacillus</i>			×
			Staphylococcaceae	<i>Staphylococcus</i>	×	×	×
		Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	×	×	
			Streptococcaceae	<i>Streptococcus</i>	×	×	
Patescibacteria	Saccharimonadia	Saccharimonadales	Uncharacterized	Uncharacterized	×	×	
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Enterobacter</i>	×	×	×
				<i>Erwinia</i>	×	×	×
				<i>Klebsiella</i>	×	×	
				<i>Pantoea</i>	×	×	×
				<i>Pluralibacter</i>	×	×	
				<i>Providencia</i>	×	×	
				<i>Pseudocitrobacter</i>	×	×	
				<i>Rosenbergiella</i>	×	×	×
		Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>			×
				Uncharacterized			×
			Pseudomonadaceae	<i>Pseudomonas</i>			×
		Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	×	×	
				<i>Vulcaniibacterium</i>			×

were identified in the current analysis and were classified into five phyla, five classes, nine orders, 11 families, and 19 genera (Table 1). Most of the detected taxa belonged to the Proteobacteria.

Effect of geographic origin on gut symbiotic community

Population 1 was mainly dominated by Gammaproteobacteria (97.6%) (Figures 1A and S1), with *Erwinia* being the most abundant genus with relative frequencies greater than 90% in the majority of the samples, followed by *Klebsiella*, *Pantoea*, *Rosenbergiella*, *Enterobacter*, *Pluralibacter*, *Providencia*, and *Pseudocitrobacter* also belonging to the Enterobacteriaceae (Figure 1B). The rest of the OTUs exhibited frequencies lower than 1% in all samples. The detailed frequencies of all detected genera are presented in the supplementary Excel file S1.

The predominant class in P2 was also Gammaproteobacteria (81.9%) followed by Deinococci (8.4%), Actinobacteria (6.5%), and Bacilli (3.2%) (Figures 2A and S1). *Erwinia* was detected in all samples (Figure 2B), ranging from 31.9 to 96.5% with the exception of the one 1-day female sample (5.7%). *Enterobacter*, *Pantoea*, and *Rosenbergiella* were the other Enterobacteriaceae members in P2 with frequencies lower than 1% in most of the samples. *Acinetobacter* (4.7%), *Pseudomonas* (5.1%), and *Vulcaniibacterium* (23%) were the other Gammaproteobacteria detected. *Meiothermus* (8.4%) and *Cutibacterium* (Propionibacteriaceae) (6.5%) were present in all

samples of P2. Two members of the Bacillales order, *Geobacillus* and *Staphylococcus*, were also detected in lower frequencies. The detailed frequencies of all detected genera are shown in the supplementary Excel file S1.

Samples of P1 exhibited similar species diversity and richness compared to P2 (Figure S2). *Erwinia* was the most dominant genus in both geographic regions with a higher average frequency in P1 (85.1%) in comparison to P2 (67.7%). Regarding beta-diversity, a non-metric multidimensional scaling (NMDS) ordination plot of microbial community structure revealed a clear distinction between the bacterial communities of the two studied populations (Kruskal-Wallis rank sum test: $P < 0.05$; Figure 3A). Visualization of all analyzed samples in a NMDS ordination plot also revealed the clustering of all samples of P2 close to each other and separated from all the samples of P1 ($P < 0.05$; Figure 3B). Therefore, the geographic origin of the flies is a factor that significantly contributes to the olive fruit fly gut microbiota structure.

A more detailed beta-diversity analysis was performed to clarify which developmental stages contribute to the differences between the two geographical origins. A NMDS ordination plot revealed no clustering of larval samples of P2 vs. pupal samples of P1 ($P > 0.05$; Figure S3A). Similarly, visualization of 1-day-old ($P > 0.05$; Figure S3B) or 5-day old adults ($P > 0.05$; Figure S3C) in a NMDS ordination plot revealed no clear clustering of samples from P1 vs. P2.

Figure 1 Relative abundance (RA) of the major bacteria (A) classes and (B) genera in pupae and 1- (1d), 5- (5d), and 15-day-old (15d) adult males (M) and females (F) of olive fruit fly populations 1 (P1) and 1A (P1A; i.e., P1 flies treated with antibiotics). Only classes and genera with RA>0.05 in at least one sample are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

Visualization of 15-day-old adult samples in the NMDS ordination plot revealed a clustering of P1 vs. P2 ($P<0.05$; Figure S3D).

Effect of antibiotic treatment on gut symbiotic community

Samples of P1 exhibited similar species diversity and richness compared to P1A ($P>0.05$; Figure S2). *Erwinia* was the most dominant genus in both populations (85.1 and 88.4%, respectively). NMDS ordination plot indicated that there are no differences between P1 and P1A regarding the microbiome profiles of 5- or 15-day-old adults as the samples from P1 are not clustered vs. the respective samples of P1A ($P>0.05$; Figure 3C,D). Slight differences that were not statistically significant included the reduction of genera that already had low frequencies in the samples that were fed with normal diet without antibiotics. More specifically, *Streptococcus* was reduced from 3.4 to 0.1%

and the uncharacterized member of the Saccharimonadales order was also reduced from 3 to 0% in 5-day-old males that were fed with adult food containing streptomycin.

Effect of gender on gut symbiotic community

The comparison of male and female samples revealed no statistically significant differences. The NMDS ordination plot failed to show any clustering of male vs. female adults ($P>0.05$; Figure S4). Thus, the sex of the fly does not influence the composition of the gut microbiota.

Effect of age and developmental stage on gut symbiotic community

The overall comparison of the alpha-diversity indices – richness (Figure S5A), Shannon (Figure S5B), and Simpson (Figure S5C) – of samples of different age or developmental stage in all analyzed populations showed

Figure 2 Relative abundance (RA) of the major bacteria (A) classes and (B) genera in larvae and 1- (1d), 5- (5d), and 15-day-old (15d) adult males (M) and females (F) of olive fruit fly population 2 (P2). Only classes and genera with RA>0.05 in at least one sample are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

no differences related to the age of the flies in the samples ($P>0.05$). NMDS ordination plot of the microbial profiles demonstrated no clustering among the various developmental stages in P2 ($P>0.05$). There was only a clear grouping of 15-day-old flies against both 1-day-old ($P<0.05$; Figure S6A) and 5-day-old flies ($P<0.05$; Figure S6B) in P1.

Discussion

The present study analyzes the gut microbiota profiles of two *B. oleae* populations from different geographic regions before and after their exposure to laboratory rearing and artificial adult diets with or without antibiotic treatment. Our study allowed the identification of a wider number of genera of bacteria that could not be detected in previous studies because of their lower frequencies or their inability to grow in selective media. It also revealed slight differences between populations that could not be unraveled

otherwise. Our data confirmed that *Erwinia* is the most predominant taxon in wild olive fruit fly populations, in accordance with a number of previous studies on wild populations (Capuzzo et al., 2005; Estes et al., 2009; Ben-Yosef et al., 2015).

In addition to *Erwinia*, a wide number of other genera belonging to the Enterobacteriaceae were identified in our samples including *Providencia*, *Enterobacter*, *Pantoea*, and *Klebsiella*. Our analysis further detected several genera in low frequencies (<3%) that had never been identified in *B. oleae*. Three of these new genera – *Pluralibacter*, *Pseudocitrobacter*, and *Rosenbergiella* – belong to the Enterobacteriaceae. *Pluralibacter* was recently differentiated from the *Enterobacter* genus (Brady et al., 2013) and was also found in the sand fly *Phlebotomus chinensis* Newstead (Li et al., 2016), the Mediterranean fruit fly (medfly), *C. capitata* (Papanicolaou et al., 2016), and the mosquito *Anopheles albimanus* Wiedemann (Dada et al., 2018). In contrast, *Pseudocitrobacter* and *Rosenbergiella* have never

Figure 3 (A) Nonmetric multidimensional scaling (NMDS) plot of bacterial communities based on relative abundances of operational taxonomic units (OTUs) in the gut of olive fruit fly samples originated from populations 1 (P1) and 2 (P2) ($P < 0.05$). (B) Multidimensional scaling (MDS) of bacterial communities based on relative abundances of OTUs in the gut of all the tested olive fruit fly samples ($P < 0.05$). (C) NMDS plot of microbial profiles of 5-day-old (5d, fed) adult olive fruit fly samples from P1 against 5-day-old adult samples of P1A (i.e., P1 flies treated with antibiotics; $P > 0.05$). (D) NMDS plot of microbial profiles of 15-day-old (15d, fed) adult olive fruit fly samples from P1 against 15-day-old adult samples of P1A ($P > 0.05$). 1d: 1-day-old adult olive fruit flies (not fed). 'd' indicates the stress value for the NMDS plot. 'corr. P-value' indicates the pairwise test significance values obtained after correction for multiple testing using the Benjamini–Hochberg method. [Colour figure can be viewed at wileyonlinelibrary.com]

been reported as insect gut microbes before. Other members of Gammaproteobacteria detected by our analysis, such as *Acinetobacter*, *Pseudomonas*, and *Stenotrophomonas*, have been previously reported in *B.*

oleae (Stamopoulos & Tzanetakis, 1988; Ben-Yosef et al., 2015; Blow et al., 2016), but it is the first time that *Vulcaniibacterium* is detected in an insect. Members of the Bacilli class such as *Staphylococcus*, *Enterococcus*, and

Streptococcus have also been detected in previous studies except for *Geobacillus* that is reported for the first time. The genera *Deinococcus* and *Meiothermus* (phylum Deinococcus–Thermus) were also identified for the first time in olive fruit fly by our study as well as the genus *Cutibacterium* (phylum Actinobacteria). It is worth noting that *A. tropicalis*, which was previously reported in Greek populations of olive fruit fly, was not detected in the samples tested in the present study (Kounatidis et al., 2009). Our data confirm the presence of a broad gut microbial diversity in the olive fruit fly. The presence of a great variety of symbiotic organisms has also been observed in NGS studies in (other) Tephritidae fruit flies and other insects (Deutscher et al., 2018; Zhao et al., 2018) and is believed to play an important role in promoting the insect fitness by providing nutrition, protection against natural enemies, and detoxification of insecticides and other toxins (reviewed in Douglas, 2015).

Samples originating from different geographical regions shared a few genera such as *Erwinia*, *Enterobacter*, *Pantoea*, *Rosenbergiella*, *Cutibacterium*, and *Staphylococcus*. However, geographical origin and environmental habitat seem to cause differences between the two populations in our analysis. *Klebsiella*, *Pluralibacter*, *Providencia*, *Pseudocitrobacter*, *Deinococcus*, *Enterococcus*, *Streptococcus*, and *Stenotrophomonas* were only detected in P1 that originated from Volos (mainland Greece), whereas *Acinetobacter*, *Pseudomonas*, *Vulcaniibacterium*, *Meiothermus*, and *Geobacillus* were only present in P2 from Crete (an island in southern Greece). These differences could be related to the different climate conditions in the regions. For example, *Meiothermus* sp., that was only identified in samples from Crete, has been detected in warm, nutrient-poor environments (Masurat et al., 2005) and is known to produce restriction endonucleases that are more resistant to extreme temperature and pH conditions (Gupta et al., 2012). Therefore, *Meiothermus* might promote the fitness of the olive fruit fly by increasing the thermostability in southern regions, such as Crete, where temperature is usually higher. Another factor that could affect the microbiome composition is the availability of bacteria in the local environment. Insect guts can be colonized by bacteria acquired from the environment, and although colonization can be selective, the composition of bacteria in the local food resources is a major determinant of the community profile (reviewed in Engel & Moran, 2013). Furthermore, genera that were identified in only one population in our study, such as *Enterococcus*, *Staphylococcus*, and *Providencia*, had very low abundancies (1–3%) and have been identified in only one or two previous studies (reviewed in Estes et al., 2011). This also supports the

hypothesis that these bacteria are probably transiently acquired from the local food resources.

All samples regardless of their geographic origin, developmental stage, age, gender, or antibiotic treatment of the adult flies contained *Erwinia* as the most dominant OTU. The average frequency of *Erwinia* was somewhat higher, but not statistically significant, in P1 (85.1%) compared to P2 (67.7%) and it was not affected by the antibiotic treatment (88.4% in P1A). However, *Erwinia* levels were somewhat lower in the first developmental stages (34.4% in larvae, 39.9% in pupae) and 1-day-old adults in comparison to older adults (5 and 15 days old) where they reached frequencies up to 99%. Although *Erwinia* is still the most dominant OTU detected, in the first developmental stages, there is a more balanced distribution among this genus and other genera such as *Klebsiella* and *Pantoea* in P1 or *Meiothermus* and *Cutibacterium* in P2. The levels of these genera seem to decrease with age, allowing the increase of *Erwinia* that gradually dominates the insect gut.

Candidatus E. dadicola is the major endosymbiont of *B. oleae*. It has been detected in all wild olive fruit fly populations in previous studies and is the most abundant bacterial species in all developmental stages of the fly. It was recently shown that *Ca. E. dadicola* enables *B. oleae* larvae to overcome the hostile environment of the unripe olive fruits, while it allows the adults to exploit intractable sources of nitrogen (Ben-Yosef et al., 2014, 2015). Previous studies demonstrated that laboratory adaptation of olive fruit flies leads to loss of *Erwinia* and its substitution by other members of the Gammaproteobacteria such as *Morganella* (Estes et al., 2011) or *Providencia* and *Acinetobacter* (Ben-Yosef et al., 2015). However, laboratory populations analyzed in previous studies all had adapted to artificial conditions for many generations. Replacement of olive fruits with artificial larval diet is probably the reason why *Ca. E. dadicola* is substituted by other bacteria. None of these previous studies, according to our knowledge, investigated whether the loss of *Erwinia* happens immediately after the rearing of wild adults in artificial diet, or whether it was due to the rearing of the next-generation larvae in artificial larval diet. Our results demonstrate that rearing of adults that emerged from wild larvae or pupae in artificial adult diet does not affect the overall frequency of *Erwinia* in the first laboratory-reared generation, whether antibiotics are used in the adult diet or not. On the contrary, *Erwinia* frequencies increase in 5- and 15-day-old adults despite the fact that they have been reared on artificial adult diet (either with or without antibiotics). The adult flies in our study emerged from wild larvae that were fed with olives in the field. These adults were fed with artificial adult diet, but our study does not include feeding

of larvae with artificial diet. Therefore, there is no replacement of the hostile phenolic environment of the olive with artificial larval diet which probably explains why there is no decrease in *Ca. E. dacicola* relative abundances in the adult stages. Our initial experimental goal was to monitor the laboratory adaptation of these two populations of *B. oleae* over several generations. Unfortunately, both of them collapsed after the first generation in the laboratory. So, whether the loss of *Erwinia* happens directly after the first generation of rearing in artificial larval diet or whether it takes several generations to decrease *Erwinia* abundance remains to be investigated. Furthermore, addition of streptomycin to the adult diet did not cause any significant change in the gut community of the adults with the exception of *Streptococcus* and *Saccharimonadales* that were only reduced in 5-day-old males. Although we could not monitor the potential changes in the overall gut community for more than one generation due to colony collapse, it is obvious that a single generation is not enough to significantly alter the insect gut microbiome.

Laboratory rearing of *B. oleae* most likely leads to the substitution of *Erwinia* with other genera consisting mostly of members of the Enterobacteriaceae. The influence of these alterations on the overall fitness of the insect varies depending on which bacterium becomes most abundant. For instance, *Morganella* is believed to be potentially pathogenic (Estes et al., 2011) whereas *Enterobacter* and *Klebsiella* have various beneficial associations with other insects (Augustinos et al., 2015; Kyritsis et al., 2017). Our study provided more information about genera such as *Enterobacter*, *Klebsiella*, and *Pseudomonas* that already occur in the early developmental stages of wild *B. oleae* populations. These genera can be used for the manipulation of the gut microbiome composition in the olive fruit fly laboratory strains and provide the opportunity to direct the substitution of *Erwinia*, that will nevertheless happen under artificial rearing conditions, by beneficial bacteria that already exist naturally in *B. oleae*. Such early attempts have been made in *B. oleae* for *Pseudomonas* sp. and indicated that the bacterium might enhance egg production by releasing amino acids required for egg maturation (reviewed in Estes et al., 2011). Similar studies in the medfly indicated that *Enterobacter* sp. improved pupal and adult productivity and *Klebsiella oxytoca* (Flügge) Lautrop affect the duration of the immature developmental stages and the flight ability of the medfly adults (Augustinos et al., 2015; Kyritsis et al., 2017). Given these previous indications, *Enterobacter* sp. and *Klebsiella* sp. derived from the olive fruit fly gut could be used to substitute *Ca. E. dacicola* in laboratory strains and produce similar results with the medfly. This beneficial manipulation could be a great contribution to the fine-tuning of the

SIT technology for olive fruit fly and so to make possible its incorporation as a component of an area-wide IPM strategy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Relative abundance (RA) of the major taxa in olive fruit fly populations 1, 1A, and 2. Only taxa with RA>0.05 in at least one sample are shown.

Figure S2 (A) Richness, (B) Shannon diversity, and (C) Simpson diversity indices of olive fruit fly populations 1, 1A, and 2.

Figure S3 Nonmetric multidimensional scaling (NMDS) plot of gut microbial profiles of olive fruit fly samples from (A) larvae of population 2 (P2) against pupae of population 1 (P1), (B) 1-day-old (1d) adults of P2 against 1d adults of P1, (C) 5-day-old (5d) adults of P2 against 5d adults of P1, and (D) 15-day-old (15d) adults of P2 against 15d adults of P1 (all comparisons: $P>0.05$)

Figure S4 Nonmetric multidimensional scaling (NMDS) plot of gut microbial profiles of olive fruit fly male samples against female samples ($P>0.05$).

Figure S5 (A) Richness, (B) Shannon diversity, and (C) Simpson diversity indices of larvae, pupae, and 1- (1d), 5- (5d), and 15-day-old (15d) adults of olive fruit fly populations 1 (P1), 1A (P1A; i.e., P1 flies treated with antibiotics), and 2 (P2) ($P>0.05$). The numbers in parentheses indicate the number of samples used in the analysis for each development stage.

Figure S6 Nonmetric multidimensional scaling (NMDS) plot of gut microbial profiles of olive fruit fly samples of (A) 1-day-old (1d) against 15-day-old (15d) adults of population 1 (P1) and (B) 5-day-old (5d) against 15d adults of P1 (both $P<0.05$).

Table S1 Number of replicates of the olive fruit fly (*Bactrocera oleae*) samples, each replicate containing the guts of five individual flies

Table S2 Mean (and standard error; derived from the three biological replicates per sample) number of reads and alpha-diversity indices of the gut microbiota in all analyzed olive fruit fly samples

Supplementary Excel file S1 Relative abundance (RA) of the different operational taxonomic units (OTUs) for each olive fruit fly sample. RA is presented at the genus level.