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Manipulation of insect gut microbiota towards the improvement of *Bactrocera oleae* artificial rearing

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Abstract

Bactrocera oleae (Rossi) (Diptera: Tephritidae) is the main pest of olive trees (*Olea europaea* L.), causing major damages in olive crops. Improvement of mass rearing is a prerequisite for the successful development of large-scale sterile insect technique (SIT) applications. This can be achieved through the enrichment of artificial diets with gut bacteria isolates. We assessed the efficiency of three gut bacteria previously isolated from *Ceratitis capitata* (Wiedemann), and four isolated from *B. oleae*, as larval diet additives in both live and inactivated/dead forms. Our results showed that dead *Enterobacter* sp. AA26 increased pupal weight, whereas both live and dead cells increased pupal and adult production and reduced immature developmental time, indicating that its bacterial cells serve as a direct nutrient source. Live *Providencia* sp. AA31 improved pupal and adult production, enhanced male survival under stress conditions, and delayed immature development. Dead *Providencia* sp. AA31, however, did not affect production rates, indicating that live bacteria can colonize the insect gut and biosynthesize nutrients essential for larval development. Live and dead *Bacillus* sp. 139 increased pupal weight, accelerated immature development, and increased adult survival under stress. Moreover, live *Bacillus* sp. 139 improved adult production, indicating that *Bacillus* cells are a direct source of nutrients. Dead *Serratia* sp. 49 increased pupal and adult production and decreased male survival under stress conditions whereas live cells decreased insect production, indicating that the live strain is entomopathogenic, but its dead cells can be utilized as nutrient source. *Klebsiella oxytoca*, *Enterobacter* sp. 23, and *Providencia* sp. 22 decreased pupal and subsequent adult production and were harmful for *B. oleae*. Our findings indicate that dead *Enterobacter* sp. AA26 is the most promising bacterial isolate for the improvement of *B. oleae* mass rearing in support of future SIT or related population suppression programs.

Introduction

The study of the microbiome has attracted the attention of the insect research community, with several studies revealing that symbiotic microbes can benefit several functions of their insect hosts and enhance their fitness (Bourtzis & Miller, 2003, 2006, 2008; Zchori-Fein & Bourtzis, 2011; Engel & Moran, 2013). Symbionts can be categorized into obligatory or primary – both the insect and the symbiont are fully dependent on each other (Baumann, 2005) – and facultative or transient – erratically distributed and not required for the host (Moran et al., 2008). Symbiotic bacteria that form obligate relationships with their host are usually unculturable endosymbionts and have generally reduced genomes compared to their free-living relatives (Charles & Ishikawa, 1999; Akman & Aksoy, 2001; Moran & Mira, 2001; Sun et al., 2001; Wernegreen et al., 2003; McCutcheon & Moran, 2012; Wernegreen, 2015, 2017).

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Important microbes are the gut-associated symbionts that reside in the insect digestive system. These symbionts facilitate insect nutrition through their involvement in nitrogen fixation (Ben-Yosef et al., 2014), vitamin synthesis, and carbon metabolism (Behar et al., 2005; Bourtzis & Miller, 2008). They also positively affect reproductive and oviposition behavior (Jose et al., 2019) and foraging behavior (Akami et al., 2019), as well as the host defense system (Ben-Yosef et al., 2015), detoxification processes, and insecticide resistance (Cheng et al., 2017; Guo et al., 2017).

The beneficial effects of gut microbiota on the fitness of their hosts could be utilized for the enhancement of insect pest control strategies that depend on large-scale insect production, such as the sterile insect technique (SIT) (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; Khaeso et al., 2018). The SIT is based on the mass rearing and sterilization through irradiation of male insects that are subsequently released in the field, where they compete with wild males for mating with wild females. As a result, the natural insect population in the field declines, as the offspring of the irradiated males are not viable due to the irradiation-induced chromosomal breaks that cause dominant lethal mutations in the sperm of the released males (Knipling, 1955; Dyck et al., 2005). Cost-effective mass production of insects with high biological quality is essential for the efficient application of SIT. Recent studies have focused on the improvement of life-history traits under mass rearing for SIT applications mostly in the tephritid flies (Diptera) *Ceratitis capitata* (Wiedemann), but also in *Bactrocera oleae* (Rossi), *Bactrocera dorsalis* (Hendel), *Bactrocera tryoni* (Froggatt), *Zeugodacus tau* (Walker), and *Anastrepha obliqua* (Macquart). These studies revealed that the incorporation of gut bacteria in larval or adult artificial diets can positively affect pupal weight (Hamden et al., 2013; Khaeso et al., 2018), adult size (Hamden et al., 2013), survival ability (Behar et al., 2008; Gavriel et al., 2011), mating competitiveness (Niyazi et al., 2004; Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013), flight ability (Kyritsis et al., 2017), pupal and adult productivity (Augustinos et al., 2015), immature development duration (Augustinos et al., 2015; Kyritsis et al., 2017; Khaeso et al., 2018), female fecundity (Sacchetti et al., 2014), and oviposition behavior (Jose et al., 2019).

Bactrocera oleae, the olive fruit fly, is the major pest of olive fruit orchards and its larvae cause huge damage to the quality of olive oil (Levinson & Levinson, 1984; Manousis & Moore, 1987). Despite the successful population management of *C. capitata* through SIT, the first attempts to apply SIT to the olive fruit fly were unsuccessful (Economopoulos, 1972, 1977; Economopoulos et al., 1976). No further progress has been achieved in the field due to the fact that mass rearing of the olive fruit fly is labor-intensive and not cost-efficient (Estes et al., 2011). Recent studies on the development of more efficient artificial rearing methodologies considerably improved the egg collection system and the adaptation of wild flies to laboratory conditions (Ahmad et al., 2014, 2016, 2018). Although current artificial diets support larval development, the quality and survival of the produced insects are still inconsistent (Estes et al., 2011; Ras et al., 2017). The major reason for the mass rearing inefficiency is the monophagous nature of the fly larvae, which obstructs the development of an adequate artificial larval diet (Manoukas, 1975; Estes et al., 2011; Ras et al., 2017). It is crucial to improve artificial diets, which may be achieved with bacterial enrichment of laboratory diets, as has been done with *C. capitata*.

Bactrocera oleae symbiotic relationship was initially examined by Petri (1909), who suggested that the associated bacteria were *Pseudomonas savastanoi*. Stammer (1929) described the vertical transmission of the gut microbes and Girolami (1973) studied the alimentary canal of the olive fruit fly and other fruit flies and identified morphological adaptations that could support the presence of associated bacteria. Other studies demonstrated that the lack of gut symbionts due to antibiotic treatment inhibits larval development (Hagen, 1966; Hagen & Tassan, 1972; Tzanakakis & Stavrinides, 1973; Lambrou & Tzanakakis, 1978). More recent studies used molecular techniques and identified *Candidatus* Erwinia dacicola (Capuzzo et al., 2005) as the major, co-evolved, obligate symbiont in wild populations, which could not be detected by previous studies due to its inability to grow in artificial bacteria media (Sacchetti et al., 2008; Estes et al., 2009, 2012; Savio et al., 2011; Koskinioti et al., 2019). *Candidatus* E. dacicola counteracts the deleterious effects of oleuropein – a toxic phenolic glycoside – and allows larval development in the hostile environment of unripe olives (Ben-Yosef et al., 2015). It is also significant for the utilization of non-essential amino acids and urea as a nitrogen source (Ben-Yosef et al., 2014). Other less abundant genera such as *Enterobacter* sp., *Providencia* sp., *Klebsiella* sp. (Manousis & Ellar, 1988; Augustinos et al., 2019; Koskinioti et al., 2019), *Acetobacter tropicalis* (Kounatidis et al., 2009), *Pantoea* sp. (Ben-Yosef et al., 2015; Koskinioti et al., 2019), *Pseudomonas* sp. (Yamvrias et al., 1970; Manousis & Ellar, 1988; Belcari et al., 2003; Sacchetti et al., 2008; Koskinioti et al., 2019), *Stenotrophomonas* sp. (Blow et al., 2016), *Tatumella* sp. (Blow et al., 2019), and *Morganella*

sp. (Augustinos et al., 2019) have also been identified in the olive fruit fly gut but their role in the fly's biology is not fully known yet.

Laboratory adaptation and rearing on artificial diets leads to loss of *Ca*. E. dacicola, decrease of bacterial diversity, and gut colonization by other species (Estes et al., 2009, 2012; Kounatidis et al., 2009). It seems that *Ca*. E. dacicola is only essential in natural populations and laboratory strains that feed on olive fruits and is replaced by other transiently acquired bacteria during feeding on artificial diets (Sacchetti et al., 2008). Some of these bacteria, such as *Morganella morganii*, are potentially pathogenic and might be the reason for *B. oleae* laboratory rearing inefficiency (Konstantopoulou et al., 2005; Kounatidis et al., 2009; Estes et al., 2011; Augustinos et al., 2019). Reintroduction of *Ca*. E. dacicola in laboratory strains could improve rearing, but the uncultivable nature of the symbiont renders it impossible to be used as probiotic supplement in mass rearing. The alternative of using crushed wild *B. oleae* guts as larval additives would be labor-intensive and the collection of adequate wild material for mass rearing purposes would be impossible. However, the targeted replacement of the insect gut microbiota with cultivable bacterial isolates originating from wild *B. oleae* populations or other bacteria known to benefit artificial rearing in other fruit flies could improve olive fruit fly rearing. Up to now, the functional role of gut symbionts on the fly's life-history traits was investigated by comparing asymbiotic flies (fed with adult diets containing antibiotics that suppress or remove the gut symbiotic microbiome) with either non-treated flies (symbiotic flies) (Ben-Yosef et al., 2010, 2014, 2015) or flies fed with adult diet enriched with certain bacterial isolates (Sacchetti et al., 2014; Jose et al., 2019). None of these studies assessed the utilization of gut microbes as additives to the larval diet of the olive fruit fly. In the current study, we evaluated the effect of bacteria-enriched larval diets on *B. oleae* life-history traits related to insect rearing and SIT application, using four bacterial isolates originated from *B. oleae* and three bacterial isolates from *C. capitata* that demonstrated beneficial probiotic behavior in previous medfly studies (Ben Ami et al., 2010; Augustinos et al., 2015; Kyritsis et al., 2017).

Materials and methods

Bactrocera oleae populations and rearing conditions

The *B. oleae* strain 'Olive-lab' (OL), used for the larval diet enrichment experiments, originated from a stock in the Department of Biology, 'Demokritos' Nuclear Research Centre, Athens, Greece, and has been maintained at the Insect Pest Control Laboratory (IPCL) for 177 generations (Ahmad et al., 2016). Adults were kept in laboratory cages (40 × 40 × 40 cm) with three net sides and a paraffin-covered fine mesh for oviposition on the fourth side and were provided with water and standard artificial adult diet consisting of 75% sugar, 19% hydrolyzed yeast, and 6% egg yolk powder, under constant environmental conditions at 25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod. Wild olive fruit flies were collected from infested olives coming from Spain. Upon emergence, wild adult flies were kept in laboratory cages and provided with sterile water and standard artificial adult diet (no antibiotics) until the day of gut dissections for the isolation of gut bacteria.

Isolation of gut bacteria and bacterial colony characterization

Three replicates of five pooled guts from teneral, 5-, and 15-day-old adults (males or females) and third instars of the wild olive fruit flies were collected. All individuals were disinfected in 70% ethanol and washed in sterile 1× phosphate buffer saline (PBS) prior to dissection. Gut dissections were performed in sterile 1× PBS and the guts were collected in 1.5-ml Eppendorf tubes containing 200 µl sterile Luria-Bertani (LB) medium (Sigma-Aldrich, St. Louis, MO, USA) and mechanically crushed using pestles. The homogenate was serially diluted and plated on three types of agar media, one non-selective (LB agar plates; Sigma-Aldrich) and two types of selective medium [ChromoCult (Merck, Darmstadt, Germany) and xylose lysine deoxycholate agar (Sigma-Aldrich)]. Duplicate plates were incubated at 25 and 37 °C. All sample treatments were performed in three replicates. In total 172 well-isolated colonies were chosen for further analysis from all sample treatments, based on colony morphology and representing all sample treatments. Three rounds of streaking and isolation were performed to ensure that they represented single colonies.

Bacterial colony characterization was performed by sequencing the 16S *rRNA* gene. 16S *rRNA* universal bacterial primers 27F/1492R (Edwards et al., 1989; Weisburg et al., 1991; Reed et al., 2002) were used to perform polymerase chain reactions (PCRs) using a few bacteria from each colony that were suspended in 50 µl of PCR reaction [25 µl of Qiagen 2× Taq mix, 0.3 µl (100 µM) of each primer]. The PCR conditions were: initial denaturing step of 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; final extension at 72 °C for 10 min. Five microliters of each reaction were electrophoresed on 1.5% agarose gels. The amplicons were purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). Purified DNA was sequenced from both ends with primers 27F and 1492R by MWG Eurofins (Ebersberg,

Germany) and/or VBC (Vienna, Austria). The obtained sequences were compared with Ribosomal Database Project (Cole et al., 2014) for the genus characterization of the colonies. Representative colonies from all genera were selected for full-length, double-stranded 16S *rRNA* sequencing using the internal primers 519F, 596R, 960R, and 1114F (Reed et al., 2002). Visualization, data quality check, and assembly of the sequencing results were performed with SeqMan software (Lasergene v.7.0; Dnastar, Madison, WI, USA). Obtained full sequences were compared with the EzBioCloud database (Yoon et al., 2017) to identify their closely related reference bacterial strains.

Origin and phylogenetic analysis of gut bacteria used as larval diet additives

Enterobacter sp. AA26 and *Providencia* sp. AA31 strains used in this study were previously isolated from medfly (Augustinos et al., 2015). The *Klebsiella oxytoca* strain was isolated and used in a previous medfly study by Ben Ami et al. (2010). *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 were isolated in the current study from wild olive fruit flies collected in Spain as previously described.

Phylogenetic analysis was based on the 16S *rRNA* sequences from the bacteria in this study, the bacterial species showing the highest similarity with the studied bacteria according to EzBioCloud, and gut bacterial species previously studied in Tephritidae (Behar et al., 2008; Wang et al., 2014; Naaz et al., 2016). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987), the confidence of the tree topology was tested by 1 000 bootstrap replicates (Felsenstein, 1985), and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Evolutionary analyses were conducted in MEGA X software (Kumar et al., 2018). Pairwise comparisons of the 16S *rRNA* sequences between the used bacterial isolates belonging to the same genus were performed in EzBioCloud database using ChunLab's online pairwise sequence alignment tool for taxonomic purposes which generates an alignment of two sequences and a sequence similarity value that is widely used in bacterial taxonomy (Yoon et al., 2017).

Enrichment of larval diet

All bacterial strains were revived from glycerol stocks kept at −80 °C by streaking on LB agar medium plates. Single colonies were selected and inoculated in LB broth medium for rearing experiments. The revived bacterial cultures were added to the larval diet in a titer of 10^8 bacteria per g of diet (Augustinos et al., 2015). The titer for each bacterial isolate was determined by measuring the optical density (OD) of each culture. The OD required to reach the appropriate titer for each isolate was determined by bacterial colony counting of serial dilutions of an initial culture with known OD. Bacterial cultures with the appropriate OD were centrifuged and resuspended in 20 ml of LB medium, before mixing with 1 kg of conventional larval diet (containing 550 ml tap water, 20 ml extra virgin olive oil, 7.5 ml Tween 80 emulsifier, 0.5 g potassium sorbate, 2 g Nipagin, 20 g sugar, 75 g brewer's yeast, 30 g soy hydrolysate, 30 ml hydrochloric acid 2N, and 275 g cellulose powder). The same number of autoclaved (dead) bacteria was also incorporated in the diet, to test whether live bacteria have an effect through interaction with the insects or whether they only serve as nutrient source. The control treatment consisted of conventional larval diet (Tsitsipis, 1975; Ahmad et al., 2016) mixed with 20 ml of LB medium (without bacteria). The diet was prepared by hand mixing directly before the addition of fly eggs.

Egg collections

Laboratory flies laid their eggs on the paraffin-covered oviposition panel that covers one of the four vertical cage sides. The oviposition panel was carefully washed with water in the morning to remove eggs laid during the previous day. Eggs laid during the next period of 6 h by 8- to 10-day-old *B. oleae* females were collected in plastic trays by washing the oviposition panel with water, counted after removing the excess of water, and transferred directly to artificial larval diet. Three replicates were used for each treatment – live and autoclaved bacteria for each strain and the control treatment – and 500 eggs were transferred to Petri dishes containing 75 g of larval diet for each replicate. Eggs for all treatments and replicates were collected from the same generation to ensure minimum egg quality variation. After their transfer, eggs were incubated under constant environmental conditions at 22 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod.

Effect of enriched larval diet on pupal weight, pupal recovery rate, egg-to-adult, and pupa-to-adult recovery rate

The pupae obtained from the 500 incubated eggs were counted for each replicate to determine the pupation recovery percentage (= 100 × no. pupae/total no. eggs). Two days before adult emergence, pupae were collected and pupal weight (mg) was determined by individually weighing all pupae in each treatment. All collected pupae were subsequently kept in Petri dishes (70 × 15 mm) at 25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod until adult eclosion to determine the egg-to-adult recovery percentage (= 100 × no. adults/total no. eggs) and the pupa-to-adult recovery percentage (= 100 × no. adults/total no. pupae).

Effect of enriched larval diet on egg-to-adult developmental duration

The following experimental procedures were only performed for the bacterial strain treatments that yielded 100 or more fly pupae per replicate (*Enterobacter* AA26 live and dead, *Providencia* AA31 live and dead, *Bacillus* 139 live and dead, and *Serratia* 49 dead) to ensure enough material for subsequent analysis. Three replicates of 100 pupae per treatment were transferred to Petri dishes and kept at 25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod. Egg-to-adult developmental duration was determined by recording the number and sex of the emerged flies twice a day (every 12 h).

Effect of enriched larval diet on adult survival under stress conditions

Survival under stress conditions is a test of the standard FAO/IAEA/USDA (2014) quality control manual for SIT research and development applications that assesses the ability of the flies to be released to survive without food, water, and light until they become sexually mature and seek for mates. Within 4 h of adult emergence (from three replicates consisting of 100 pupae), the flies were transferred to a large Petri dish (70 × 15 mm) with a mesh-covered hole of approximately 13 mm in the center of the lid. All dishes were kept in the dark at 26 °C and 65% r.h. until death of the last fly. Dead flies were sorted by sex, counted, and removed from the Petri dishes twice a day (every 12 h) in order to determine and compare their ability to survive under food and water deprivation.

Effect of enriched larval diet on adult flight ability

Three replicates of 100 pupae per treatment were transferred within a ring of paper that was centrally placed at the bottom of a Petri dish (77 × 15 mm) 2 days before adult emergence. An opaque black plexiglass tube of 10 cm height was placed over the Petri dish according to the procedure described in FAO/IAEA/USDA (2014). Flies that emerged were removed from the vicinity of the tubes to minimize fly-back (or fall-back) into the tubes. The test lasted 1 week and took place at 25 °C, 65% r.h., L14:D10 photoperiod, and 1 500 lux light intensity over the tubes. Flight ability was determined by recording the number and sex of the flies that managed to fly out of the tube.

Statistical analysis

The effects of the various bacterial treatments on pupal weight were tested with one-way ANOVA with 'treatment' as the independent variable. A post-hoc test was used for multiple comparisons of the tested groups using Bonferroni adjustment of P-values. The effect of bacteria provision on pupal recovery percentage, egg-to-adult and pupa-to-adult recovery percentage, flight ability, and sex ratio was determined by binary logistic regression (BLR) analysis using Bonferroni correction to adjust the P-value for multiple comparisons. The Kaplan-Meier test was used to determine the effect of bacteria provision on the egg-to-adult developmental duration. Pairwise comparisons between treatments were tested with the Mantel-Cox log-rank test with a corrected significance threshold for multiple comparisons of the eight treatments of $\alpha = 0.05/8 = 0.00625$. Cox regression analysis was used to assess the effect of bacteria provision and fly sex on adult survival under stress conditions and pairwise comparisons between the eight treatments were tested with the Mantel-Cox log-rank test ($\alpha = 0.00625$). All datasets were analyzed in IBM SPSS v.24.0 software (IBM SPSS, Armonk, NY, USA).

Results

Characterization of the gut bacteria isolates by 16S *rRNA* sequencing

The closest relatives of the gut bacterial isolates used for downstream larval diet enrichment experiments were identified by EzBioCloud analysis of full-length sequences of the 16S *rRNA* gene. *Enterobacter* sp. AA26 showed 99.8% similarity with *Enterobacter hormaechei* subsp. *steigerwaltii*, *Providencia* sp. AA31 was 99.7% identical to *Providencia vermicola*, *Enterobacter* sp. 23 was 99.7% identical to *Enterobacter ludwigii*, *Providencia* sp. 22 showed 99.9% similarity with *Providencia huaxiensis*, and the closest relatives of *Bacillus* sp. 139 and *Serratia* sp. 49 were *Bacillus siamensis* (99.9% similarity) and *Serratia marcescens* subsp. *sakuensis* (99.7% similarity), respectively (Table S1). The similarity between *Enterobacter* sp. AA26 and *Enterobacter* sp. 23 according to EzBioCloud database using ChunLab's online pairwise sequence alignment tool was 98.3%, whereas the similarity of *Providencia* sp. AA31 with *Providencia* sp. 22 was 99.6% (Table S2).

Phylogenetic analysis based on 16S *rRNA* sequences from the bacteria used in this study, their closest relatives according to EzBioCloud, and gut bacterial species previously reported from Tephritidae (Behar et al., 2008; Wang et al., 2014; Naaz et al., 2016) indicated the clustering of *Enterobacter* sp. 23 with *Enterobacter cloaceae*, whereas *Enterobacter* sp. AA26 was clustered with *E. hormaechei* subsp. *steigerwaltii* in a different clade. *Providencia* sp. 22 was clustered with *P. huaxiensis*, whereas *Providencia* sp. AA31 clustered in a different clade closer to *P. vermicola*. *Bacillus* sp. 139 was grouped with *B. siamensis* and *Serratia* sp. 49 was clustered with *S. marcescens* subsp. *sakuensis* (Figure 1). Accession numbers of the bacteria from previous Tephritidae studies are listed in Table S3. Full-length sequences of the *B. oleae* gut bacteria used in this study have been deposited in GenBank database under the accession numbers MN560062–MN560065.

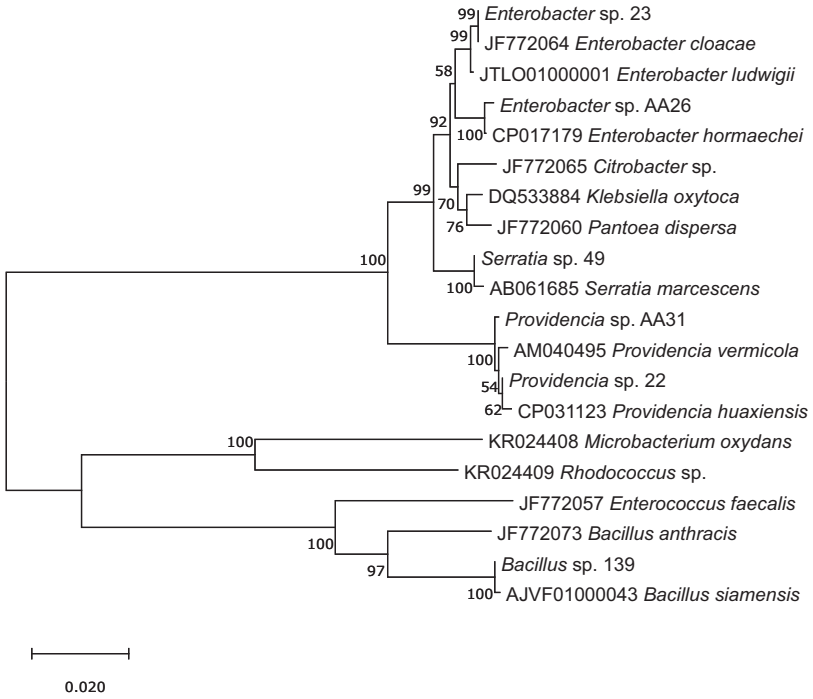

Figure 1 Neighbor-Joining phylogenetic tree based on *16S rRNA* sequences of the bacterial isolates of *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49, the bacteria species showing the highest similarity with the studied bacteria according to EzBioCloud, and gut bacterial species from Tephritidae in previous studies. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed with the Maximum Composite Likelihood method (unit: number of base substitutions per site). This analysis involved 20 nucleotide sequences. In total 1 531 positions were used in the final dataset. Evolutionary analysis was conducted in MEGA X software.

Effects of bacteria-enriched larval diet on pupal weight

Pupal weight was affected by bacterial treatment ($F_{14,4331}$ = 27.398, P<0.001; Table S4). More specifically, the addition of live *Enterobacter* sp. AA26 (mean ± SE = 6.44 ± 0.04 mg) to the larval diet caused an increase in pupal weight in comparison to both the control (6.09 ± 0.07 mg) and the autoclaved AA26 (6.04 ± 0.04 mg) (ANOVA: P<0.001; Figure 2). Dead *Providencia* sp. AA31 (6.63 ± 0.05 mg) increased pupal weight compared to both the control and the live treatment (6.24 ± 0.036 mg) (both P<0.001). Addition of live (5.78 ± 0.09 mg) and dead (6.06 ± 0.12 mg) *K. oxytoca* had no significant effect on pupal weight compared to the control treatment (P = 0.36 and 1.0, respectively). Live *Enterobacter* sp. 23 (6.56 ± 0.09 mg) increased pupal weight compared to the control treatment (P = 0.001). Live *Providencia* sp. 22 (6.53 ± 0.08 mg) increased the pupal weight compared to control (P = 0.002). Both live (6.72 ± 0.06 mg) and dead *Bacillus* sp. 139 (6.75 ± 0.06 mg) increased pupal weight (P<0.001), whereas live *Serratia* sp. 49 (5.56 ± 0.12 mg) decreased pupal weight compared to the control (P = 0.004; Figure 2). P-values for pairwise comparisons of pupal weight for all treatments are shown in Table S4.

Effects of bacteria-enriched larval diet on pupal recovery rate

Pupa recovery rate was affected by bacteria treatment (overall Wald's χ^2 = 2997.753, d.f. = 14, P<0.001; Table S5). More specifically, provision of both live (mean ± SE = 37.5 ± 0.012%) and dead *Enterobacter* sp. AA26 (48.1 ± 0.013%) increased pupation rate compared to control (21.5 ± 0.011%) (BLR: P<0.001) and the increase caused by the addition of dead *Enterobacter* sp. AA26 was stronger compared to live *Enterobacter* sp. AA26 treatment (P<0.001). Live *Providencia* sp. AA31 (50.4 ± 0.013%) improved pupal recovery compared to both the control and the dead *Providencia* sp. AA31 (24 ± 0.011%) treatment (P<0.001). On the other hand, both live (9.9 ± 0.008%) and dead *K. oxytoca* (8.1 ± 0.007%) decreased pupal recovery compared to the control treatment (P<0.001). *Enterobacter* sp. 23 (both live and autoclaved, 10.3 ± 0.008 and 5.9 ± 0.007%,

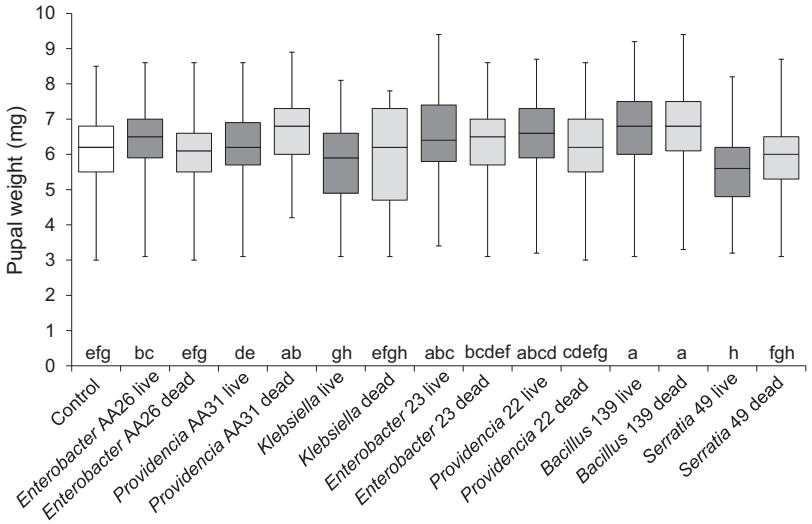

Figure 2 Effect of larval diets enriched with LB medium (without bacteria; control), *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on pupal weight of olive fruit fly. The top and bottom of the boxes represent the 25th and 75th percentiles, indicating the inter-quartile range. The horizontal line within the box represents the median value. The whiskers indicate the highest and lowest observations and define the variability outside the inter-quartile range. Treatments marked with different letters on the x-axis cause a statistically significant difference in fly pupal weight (one-way ANOVAs followed by Bonferroni test: $P<0.05$).

respectively) decreased pupation rate ($P<0.001$) and the decrease caused by dead *Enterobacter* sp. 23 was stronger compared to live *Enterobacter* sp. 23 ($P = 0.001$). *Providencia* sp. 22 (both live and dead, 10.6 ± 0.008 and $7.6 \pm 0.007\%$, respectively) also caused a decrease in pupation rate ($P<0.001$), whereas *Bacillus* sp. 139 (live and autoclaved, 18.8 ± 0.01 and $24.1 \pm 0.011\%$, respectively) had no effect compared to control ($P = 1.0$). A significant decrease in pupal recovery rate was observed by live *Bacillus* sp. 139 compared to dead *Bacillus* sp. 139 treatment ($P = 0.045$). Live *Serratia* sp. 49 ($6.9 \pm 0.007\%$) decreased pupation compared to the control treatment ($P<0.001$), whereas dead *Serratia* sp. 49 ($39.5 \pm 0.013\%$) increased pupal recovery compared to both the control and the live *Serratia* sp. 49 treatment ($P<0.001$; Figure 3). P-values for pairwise comparisons of pupation percentage for all treatments are shown in Table S5.

Effects of bacteria-enriched larval diet on egg-to-adult and pupa-to-adult recovery rate and adult sex ratio

Egg-to-adult recovery rate was affected by bacteria treatment (overall Wald's $\chi^2 = 2867.034$, d.f. = 14, $P<0.001$; Table S6). More specifically, both live and dead *Enterobacter* sp. AA26 (33.7 ± 0.012 and $41.5 \pm 0.013\%$, respectively) increased egg-to-adult recovery percentage compared to control ($17.9 \pm 0.01\%$) (BLR: $P<0.001$). The increase by dead *Enterobacter* sp. AA26 was stronger compared to live *Enterobacter* sp. AA26 treatment ($P<0.001$; Figure 4). Live *Providencia* sp. AA31 ($42.1 \pm 0.013\%$) improved egg-to-adult recovery compared to both the control and the dead *Providencia* sp. AA31 treatment ($20.3 \pm 0.01\%$) ($P<0.001$). On the other hand, both live and dead *K. oxytoca* (8.3 ± 0.007 and $7.3 \pm 0.007\%$, respectively) decreased egg-to-adult recovery compared to control ($P<0.001$). Similarly, *Enterobacter* sp. 23 (both live and autoclaved, 5.5 ± 0.007 and $4.9 \pm 0.006\%$, respectively), reduced egg-to-adult recovery compared to the control ($P<0.001$). Live and dead *Providencia* sp. 22 (9 ± 0.007 and $5.5 \pm 0.006\%$, respectively) also decreased egg-to-adult recovery compared to the control ($P<0.001$) and the decrease caused by dead *Providencia* sp. 22 was stronger compared to live *Providencia* sp. 22 ($P = 0.026$). Live and dead *Bacillus* sp. 139 (17.5 ± 0.01 and $21.1 \pm 0.011\%$, respectively) treatment had no effect on egg-to-adult recovery compared to the control ($P = 1.0$). Live *Serratia* sp. 49 ($2.3 \pm 0.004\%$) decreased egg-to-adult recovery compared to the control treatment ($P<0.001$), whereas dead *Serratia* sp. 49 ($31.3 \pm 0.012\%$) increased egg-to-adult recovery compared to both the control and the live *Serratia* sp. 49 treatment ($P<0.001$; Figure 4). P-values for pairwise comparisons of egg-to-adult recovery for all treatments are shown in Table S6.

Pupa-to-adult recovery percentage was affected by bacteria treatment (overall Wald's $\chi^2 = 241.965$, d.f. = 14, $P<0.001$; Table S7). *Enterobacter* sp. AA26, *Providencia* sp.

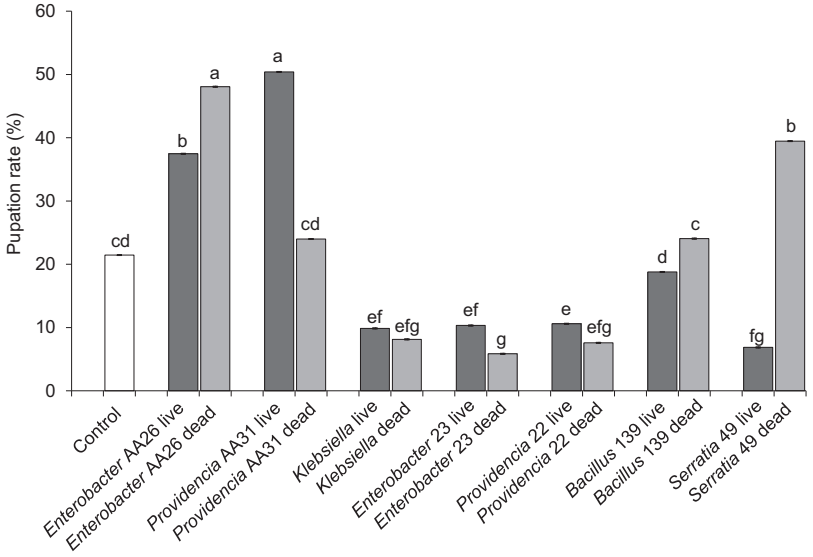

Figure 3 Effect of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (+ SEM) egg-to-pupa recovery (%; 100× no. pupae/no. eggs) of the olive fruit fly. Means capped with different letters are significantly different (BLR followed by Bonferroni test: $P<0.05$).

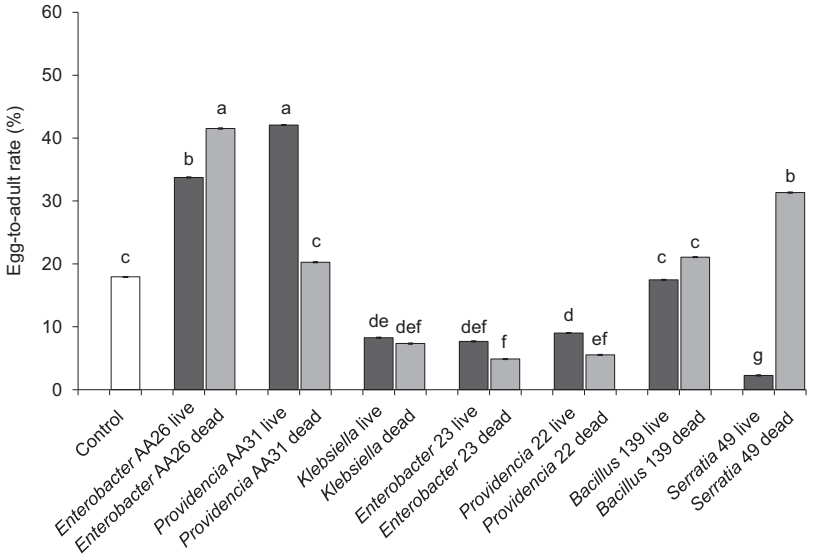

Figure 4 Effect of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (+ SEM) egg-to-adult recovery (%; 100 × no. adults/no. eggs) of the olive fruit fly. Means capped with different letters are significantly different (BLR followed by Bonferroni test: $P<0.05$).

AA31, *K. oxytoca*, *Enterobacter* sp. 23, and *Providencia* sp. 22 had no effect on pupa-to-adult emergence percentage compared to control (BLR: $P>0.05$; Figure 5, Table S7). Live *Bacillus* sp. 139 (95.3 ± 0.013%) increased pupa-to-adult recovery compared to control (82.3 ± 0.021%) ($P<0.001$) whereas live *Serratia* sp. 49 (33.6 ± 0.046%) decreased pupa-to-adult recovery percentage compared to both the control and the dead *Serratia* sp. 49 (76.4 ± 0.017%) treatment ($P<0.001$; Figure 5). P-values for pairwise comparisons of pupa-to-adult recovery for all treatments are shown in Table S7.

Adult sex ratio was not affected by bacteria treatment (overall Wald's $\chi^2 = 3.814$, d.f. = 14, P = 1.0; Figure S1). P-values for pairwise comparisons of adult sex ratio for all treatments are shown in Table S8.

Effects of bacteria-enriched larval diet on egg-to-adult developmental duration

Bacteria enrichment of larval diet with *Enterobacter* sp. AA26, *Providencia* sp. AA31, and *Bacillus* sp. 139 had significant effect on the egg-adult developmental duration for both live and autoclaved treatments compared to control (males: 28.65 days, females: 28.69 days; Table S9). More specifically, *Enterobacter* sp. AA26 accelerated adult emergence in both males (live: 27.82 days, log-rank test $\chi^2 = 59.223$; dead: 27.73 days, $\chi^2 = 63.266$) and females

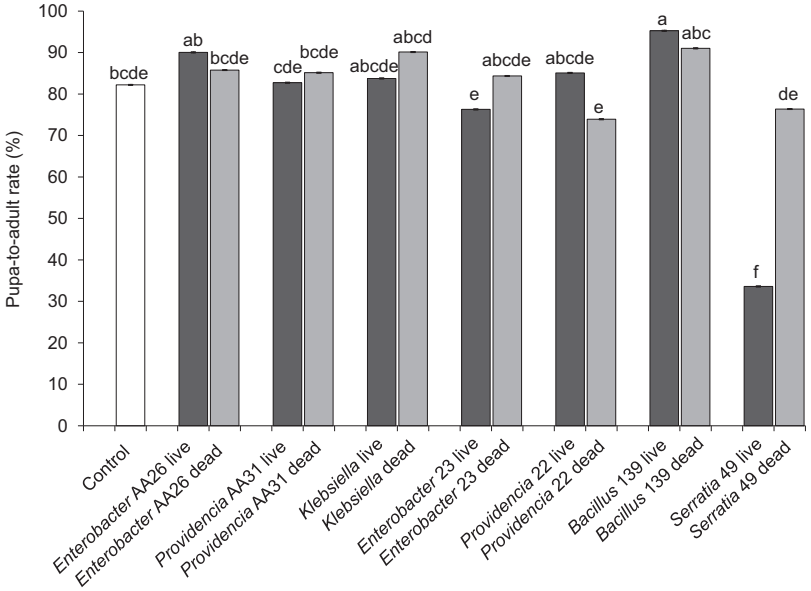

Figure 5 Effect of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (+ SEM) pupa-to-adult recovery (%; 100 × no. adults/no. pupae) of the olive fruit fly. Means capped with different letters are significantly different (BLR followed by Bonferroni test: $P<0.05$).

(live: 28.06 days, $\chi^2 = 75.112$; dead: 27.84 days, $\chi^2 = 96.451$, all $P<0.001$; Figure 6). In contrast, the addition of *Providencia* sp. AA31 delayed the emergence date in both males (live: 29.14 days, $\chi^2 = 43.240$; dead: 28.89 days, $\chi^2 = 17.986$, both $P<0.001$) and females (live: 29.21 days, $\chi^2 = 11.762$, $P = 0.001$; dead: 29.19 days, $\chi^2 = 12.392$, $P<0.001$). Incorporation of *Bacillus* sp. 139 in larval diet led to faster emergence in both males (live: 28.02 days, $\chi^2 = 33.742$; dead: 28.05 days, $\chi^2 = 48.554$) and females (live: 28.5 days, $\chi^2 = 13.353$; dead: 28.4 days, $\chi^2 = 26.823$, all $P<0.001$). Dead *Serratia* sp. 49 had no effect on the egg-to-adult developmental duration in both males (28.52 days, $\chi^2 = 2.954$, $P = 0.086$) and females (28.87 days, $\chi^2 = 1.190$, $P = 0.28$; Figure 6). P-values for pairwise comparisons of adult egg-to-adult developmental duration for all treatments are shown in Table S9.

Effects of bacteria-enriched larval diet on adult survival

Adult survival under stress conditions was affected by bacteria treatment in both males (overall Wald's $\chi^2 = 77.186$) and females ($\chi^2 = 85.748$, both d.f. = 7, $P<0.001$; Table S10). *Enterobacter* sp. AA26 (live: 37.06 h, dead: 37.45 h) had no effect on male survival compared to control (males: 39.96 h) ($P>0.00625$; Figure 7A, Table S11), but decreased adult survival duration compared to control (females: 41.19 h) in females (live: 36.77 h, $\chi^2 = 16.028$; dead: 35.3 h, $\chi^2 = 20.944$, both $P<0.001$; Figure 7B). Live *Providencia* sp. AA31 increased adult survival duration compared to the control in males (44.28 h, $\chi^2 = 10.134$, $P = 0.001$; Figure 7C) and had no effect on female survival (44.03 h, $\chi^2 = 3.216$, $P = 0.073$; Figure 7D). Dead *Providencia* sp. AA31 increased adult survival duration in both males (47.46 h, $\chi^2 = 28.828$; Figure 7C) and females (46.51 h, $\chi^2 = 14.050$, both $P<0.001$; Figure 7D). *Bacillus* sp. 139 (live and dead) increased adult survival duration compared to the control in both males (live: 46.09 h, $\chi^2 = 19.505$; dead: 44.70 h, $\chi^2 = 14.334$, both $P<0.001$; Figure 7E) and females (live: 46 h, $\chi^2 = 9.833$, $P = 0.002$; dead: 45.6 h, $\chi^2 = 14.381$, $P<0.001$; Figure 7F). Dead *Serratia* sp. 49 decreased adult survival duration compared to control in males (35.47 h, $\chi^2 = 9.353$, $P = 0.002$; Figure 7G) and had no effect on female survival (38.16 h, $\chi^2 = 6.214$, $P>0.00625$; Figure 7H). Cox regression P-values of adult survival under stress conditions for all treatments are shown in Table S10. P-values for pairwise comparisons of adult survival under stress conditions for all treatments are shown in Table S11.

Effects of bacteria-enriched larval diet on adult flight ability

Flight ability was not affected by the enrichment of larval diet with bacteria (overall Wald's $\chi^2 = 9.151$, d.f. = 7, $P = 0.24$; Figure S2, Table S12). The average percentage of fliers in the control treatment was 84.9%. *Enterobacter* sp. AA26 treatment led to 87.9 and 86.1% fliers in live and dead treatment, respectively. The flight ability of the flies fed with live and dead *Providencia* sp. AA31 was 93.3 and 90.6%, respectively. Flight ability of flies fed with live *Bacillus* sp. 139, dead *Bacillus* sp. 139, and dead *Serratia* sp. 49 was 85.4, 88.9, and 80.5%, respectively. P-values for pairwise comparisons of adult flight ability for all bacteria treatments are shown in Table S12.

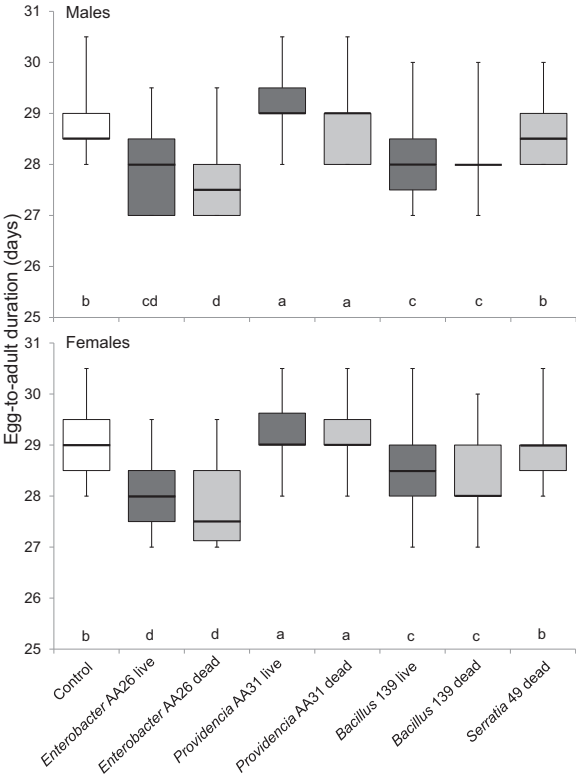

Figure 6 Effect of larval diets enriched with LB medium (without bacteria; control), *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on olive fruit fly male (top) and female (bottom) egg-to-adult developmental duration. The top and bottom of the boxes represent the 25th and 75th percentiles, indicating the inter-quartile range. The horizontal line within the box represents the median value. The whiskers indicate the value range (min-max). Treatments marked with different letters on the x-axis cause a statistically significant difference in parasitism rate (log-rank test: $P<0.003$).

Discussion

In the current study we evaluated whether seven gut bacterial isolates could function as additives in the artificial larval diet of the olive fruit fly. We used live and dead *Enterobacter* sp. AA26, *Providencia* sp. AA31, and *K. oxytoca* that were isolated from the medfly gut and showed beneficial effects on *C. capitata* rearing in previous studies (Ben Ami et al., 2010; Augustinos et al., 2015). We also used *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 isolated from the olive fruit fly gut in this study. Pupal weight, pupal recovery, egg-to-adult recovery, egg-to-adult developmental duration, and survival under water and food deprivation were affected by the bacteria-enriched larval diets and the effects of all bacteria treatments are summarized in Table 1. On the other hand, sex ratio and flight ability were not affected by any of the treatments.

Live *Enterobacter* sp. AA26 generally improves insect performance as it increases pupal weight, pupal recovery, and egg-to-adult recovery, and reduces egg-to-adult developmental duration but reduces female survival under water and food deprivation compared to the control treatment. Dead *Enterobacter* sp. AA26 has the same effects as live *Enterobacter* sp. AA26 with the exception of pupal weight, which is not affected by the dead *Enterobacter* sp. AA26 treatment. The fact that both live and dead bacterial treatments have a generally positive effect on olive fruit fly production indicates that bacterial cells might not establish in the gut but are directly consumed as food by the fly larvae, providing amino acids, nitrogen compounds, vitamins, and other nutrients that increase the weight of the pupae and the number of larvae that reach pupation, and decrease the time required for the egg-to-adult development. Adult traits, such as survival and flight ability, are not positively affected by *Enterobacter* sp. AA26 (live or dead) which further enhances the assumption that the bacteria did not colonize the fly's gut. However it has also been shown that autoclaved bacteria – also called 'paraprobiotics' – might provide health benefits to their hosts through pathways not related to nutrition by (1) modulating the immune system (compounds of the cell wall might enhance the immunological system), (2) increasing adhesion to intestinal cells which inhibits their colonization by pathogens, and (3) secreting of beneficial metabolites by the dead cells (de Almada et al., 2016). The increase of pupal and adult recovery caused by dead *Enterobacter* sp. AA26 is stronger than live *Enterobacter* sp. AA26, which indicates that using autoclaved bacteria as larval diet additives could yield more *B. oleae* insects compared to live probiotics – this would simplify the rearing process and raise fewer biosecurity concerns in future applications.

Providencia sp. AA31 increases *B. oleae* production only when used as live bacteria. It also increases male survival under water and food deprivation. Positive effects caused by the live treatment indicated that these bacteria might be able to colonize the insect gut and provide the insect with amino acids, carbohydrates, vitamins, and other nutrients. Genome sequencing of *Ca.* E. dacicola and *Enterobacter* sp. OLF – isolated from wild *B. oleae* flies – showed that both of them have multiple pathways for the biosynthesis of all essential and non-essential amino acids (Estes et al., 2018). Additionally, the same *Enterobacter* isolate can synthesize the same vitamins with *Ca.* E. dacicola and encodes several genes for nitrogen fixation (Estes et al., 2018). It is possible that other bacteria isolated from the gut of *B. oleae* or other fruit flies are also able to substitute *Ca.* E. dacicola in providing all necessary nutrients to the fly. Our findings

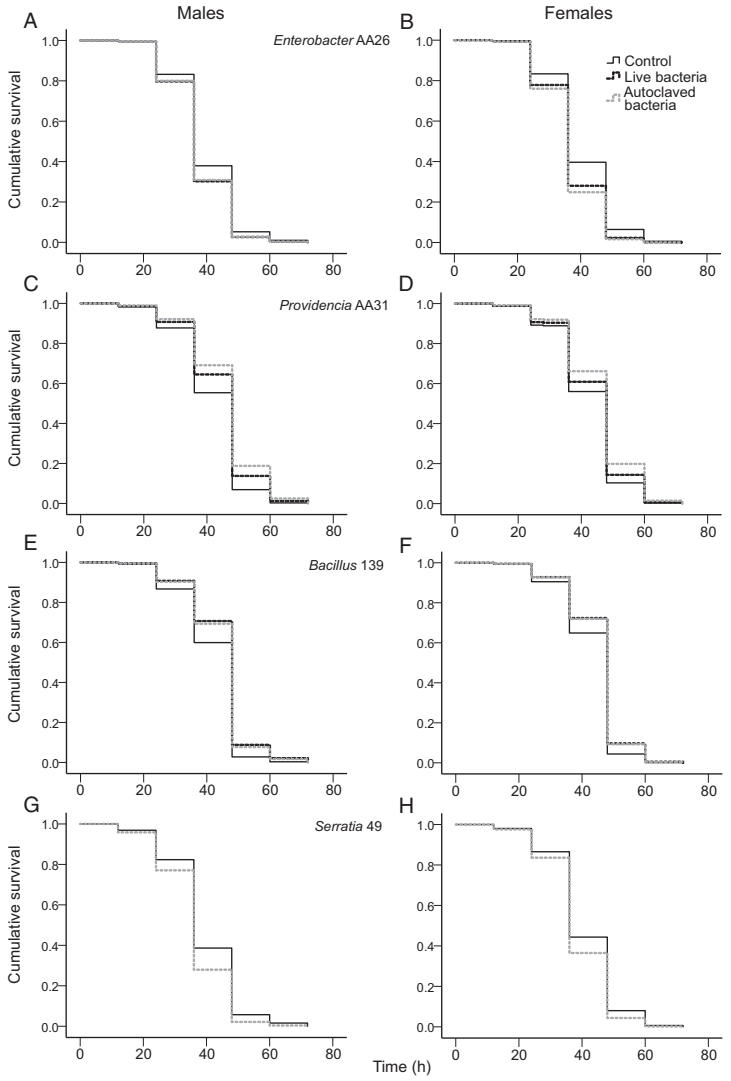

Figure 7 Male and female olive fruit fly adult survival under stress conditions, with or without (= control) live or dead (= autoclaved) bacteria incorporated in the larval diet: (A, B) *Enterobacter* sp. AA26, (C, D) *Providencia* sp. AA31, (E, F) *Bacillus* sp. 139, or (G, H) *Serratia* sp. 49.

show that *Providencia* sp. AA31 may have this ability. However, live *Providencia* sp. AA31 seems to delay egg-to-adult development but this is only a delay of approximately 12 h and might be related to the time required until colonization in the fly's gut. Alternatively, the delay of immature developments may be due to metabolites produced by the bacteria. For instance, it has been shown that a commensal gut bacterium, *Acetobacter pomorum*, produces the metabolite acetate that modulates insulin/insulin-like growth factor signaling in *Drosophila*, which is important for normal larval development (Shin et al., 2011). The metabolites potentially produced by fruit fly gut bacteria and the effect (positive or negative) they might have on the gut microbiome and the host are unknown. However, it is possible that *Providencia* sp. AA31 produces such metabolites that affect immature development. The benefits of *Providencia* sp. AA31 might also be caused by the colonization of the insect gut by *Providencia*, which might increase resistance of the insect against other entomopathogenic bacteria that are known to occupy the insect gut in laboratory strains (Estes et al., 2011; Augustinos et al., 2019). The underlying mechanisms of this colonization resistance can be nutrient competition, niche occupation, or immune priming (Engel & Moran, 2013). Dead *Providencia* sp. AA31 increases adult survival but has no effect on pupal and adult production which is more crucial for mass-rearing improvement. Therefore, only live *Providencia* sp. AA31 could be used as a probiotic supplement for the improvement of *B. oleae* rearing, which may not be applicable in real conditions.

The effect of *K. oxytoca* was generally negative, as it strongly decreased pupal recovery and subsequently egg-

Table 1 Summary of the effect of bacteria-enriched larval diets on life-history traits of the olive fruit fly compared to the control treatment

Treatment	Pupal weight	Pupal recovery	Egg-to-adult recovery	Pupa-to-adult recovery	Egg-to-adult developmental duration		Adult survival under stress	
					Males	Females	Males	Females
Enterobacter sp. AA26								
Live	Positive	Positive	Positive	=	Positive	Positive	=	Negative
Dead	=	Positive	Positive	=	Positive	Positive	=	Negative
Providencia sp. AA31								
Live	=	Positive	Positive	=	Negative	Negative	Positive	=
Dead	Positive	=	=	=	Negative	Negative	Positive	Positive
Klebsiella oxytoca								
Live	=	Negative	Negative	=	N/A	N/A	N/A	N/A
Dead	=	Negative	Negative	=	N/A	N/A	N/A	N/A
Enterobacter sp. 23								
Live	Positive	Negative	Negative	=	N/A	N/A	N/A	N/A
Dead	=	Negative	Negative	=	N/A	N/A	N/A	N/A
Providencia sp. 22								
Live	Positive	Negative	Negative	=	N/A	N/A	N/A	N/A
Dead	=	Negative	Negative	=	N/A	N/A	N/A	N/A
Bacillus sp. 139								
Live	Positive	=	=	Positive	Positive	Positive	Positive	Positive
Dead	Positive	=	=	=	Positive	Positive	Positive	Positive
Serratia sp. 49								
Live	Negative	Negative	Negative	Negative	N/A	N/A	N/A	N/A
Dead	=	Positive	Positive	=	Positive	Positive	Negative	=

N/A, not tested; =, no effect.

to-adult recovery rates, to the point that there was no adequate material for further analysis. Live and dead *Enterobacter* sp. 23 had a negative effect on *B. oleae* production as it strongly decreased pupal and adult recovery, whereas only live *Enterobacter* sp. 23 increased pupal weight. Similarly, live and dead *Providencia* sp. 22 also decreased pupal and adult recovery but only live *Providencia* sp. 22 increased pupal weight. Like *K. oxytoca,* the decrease in productivity after *Enterobacter* sp. 23 and *Providencia* sp. 22 did not allow further analysis on egg-to-adult developmental and adult survival and flight ability. The negative effects of these isolates show that all three of them are potentially pathogenic for the olive fruit fly. Interestingly, the negative effects are also extended to their autoclaved treatments. This might be explained by the presence of bacterial toxins that were ingested by the larvae during feeding. There has been evidence of thermo-stable enterotoxins that can maintain their molecular structure at high temperatures (Regenthal et al., 2017). The treatments might contain such stable bacterial toxins even though the bacteria were autoclaved. The increase in pupal weight by *Enterobacter* sp. 23 and *Providencia* sp. 22 might be explained by the small number of larvae that survived and had larger amounts of food available to consume in each replicate.

Bacillus sp. 139 had an overall positive impact as it increased pupal weight (both live and dead), did not affect pupation rate but increased pupa-to-adult recovery (only live treatment), reduced the time required for the egg-to-adult development for males and females (both live and dead treatment), and increased male and female survival under stress conditions (both live and dead treatment). Therefore, dead *Bacillus* sp. 139 could be used for the acceleration but not for the increase of *B. oleae* production and only live treatment would do both. Most of the positive effects caused by the live treatment of *Bacillus* sp. 139 can also be seen in the autoclaved treatments. This means that its bacterial cells (dead or alive) are probably directly consumed as food by the fly larvae, providing nutrients (amino acids, nitrogen compounds, vitamins, etc.) that (1) increase pupal weight leading to stronger adults with higher survival rates, and (2) decrease the time required for egg-to-adult development.

Live *Serratia* sp. 49 had an overall negative effect by decreasing pupal weight, pupal recovery percentage, egg-to-adult and pupa-to-adult recovery rate. This led to

inadequate material for further analysis and indicates that these bacterial isolates are not appropriate for larval diet enrichment applications. The negative effects might be the result of the substitution of other beneficial bacteria with *Serratia* that is unable to provide the same potential benefits provided by the previous gut microbes. Alternatively, *Serratia* sp. 49 may produce bacterial toxins that negatively affect insect health. The closest relative of our *Serratia* isolate has been identified as *S. marcescens*, an entomopathogen that is insensitive to the host's systemic immune response and kills *Drosophila* and *Rhagoletis pomonella* (Walsh) flies (Lauzon et al., 2003; Nehme et al., 2007). *Serratia* sp. 49 is probably an entomopathogen like its closer relative, which would explain the detrimental effect of the live treatment on the olive fruit fly rearing. In contrast, dead *Serratia* sp. 49 increased pupal recovery percentages and egg-to-adult recovery rate, did not affect egg-to-adult developmental duration, but decreased male longevity. The delay of immature developments may be due to metabolites secreted by the dead bacterial cells that modulate developmental pathways (Shin et al., 2011). The metabolites potentially produced by fruit fly gut bacteria and the effect (positive or negative) they might have on the gut microbiome and the host are unknown. However, it is possible that dead *Serratia* sp. 49 secretes such metabolites that affect immature development. Dead bacterial cells function as a direct source of nutrients (nitrogen, amino acids, vitamins, etc.) that improves pupal and egg-to-adult recovery. Dead *Serratia* sp. 49 could be used as a larval diet additive that increases olive fruit fly production.

Adult sex ratio was not affected by any of the bacterial isolates. This is expected because none of the tested bacteria is among the endosymbionts that are known to manipulate insect reproduction, such as *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, and *Spiroplasma* (Bourtzis & Miller, 2008; Beukeboom, 2012). Adult flight ability was also not affected by any of the treatments. It seems that the nutritional benefits provided by the bacteria added to the larval diet are related to the provision of nutrients during the larval stage and the same bacteria do not have a similar functional role in the adult stage. This could be explained by changes in the structure of the gut microbiome taking place during metamorphosis, which might lead to the elimination or change in the concentration of the bacteria that were transiently acquired during larval feeding (Engel & Moran, 2013).

Results from *Enterobacter* sp. AA26 treatment are diametrically opposed to those of *Enterobacter* sp. 23. A similar difference was observed between *Providencia* sp. AA31 and *Providencia* sp. 22. These isolates have been identified to belong to the same genus, but this does not mean that they also belong to the same species. It is possible that the two *Enterobacter* sp. isolates (or the two *Providencia* sp. isolates) are essentially different species and affect olive fruit fly biology in a completely different way. This hypothesis is also supported by the clustering of *Enterobacter* sp. 23 with *E. cloaceae*, whereas *Enterobacter* sp. AA26 is grouped with *E. hormaechei* subsp. *steigerwaltii* in a different clade of the NJ phylogenetic tree. Similarly, *Providencia* sp. 22 clustered with *P. huaxiensis* whereas *Providencia* sp. AA31 is clustered in a different clade closer to *P. vermicola*. However, 16S *rRNA* gene analysis alone is not adequate for bacteria species classification due to the presence of mosaicism, intra-genomic heterogeneity, and lack of a universal threshold sequence identity value (Rajendhran & Gunasekaran, 2011). Therefore, our analysis is actually limited to the genus level and the clustering of our isolates to specific clades of the phylogenetic tree is only an indication but not proof of species classification. The differences between the two *Providencia* sp. (or the two *Enterobacter* sp.) might be the result of genes encoding for nutrient biosynthesis in the genome of *Providencia* sp. AA31, for instance, which are not present in *Providencia* sp. 22. Alternatively, genes encoding for toxins or other harmful metabolites might be present in the genome of *Providencia* sp. 22 (or *Enterobacter* sp. 23) but not in that of *Providencia* sp. AA31 (or *Enterobacter* sp. AA26).

Enterobacter sp. AA26 was previously studied as larval diet supplement in medfly, where it also increased pupal and adult productivity and induced faster development (Augustinos et al., 2015). Although, there was no positive effect on medfly pupal weight, as is the case with the olive fruit fly, the overall positive effect is very similar between the two studies. *Enterobacter* sp. AA26 was also tested as additive to adult diets (Kyritsis et al., 2017) with no significant effect on medfly performance and as an effective protein substitute for brewer's yeast in *C. capitata* larval diet that resulted in decreased mortality of immature stages, accelerated immature development, increased pupal weight, and prolonged survival under stress conditions (Kyritsis et al., 2019). Also, biochemical and nutritional characterization of *Enterobacter* sp. AA26 demonstrated that as a probiotic strain it can provide all essential and non-essential amino acids and vitamins required for the efficient medfly mass rearing (Azis et al., 2019). All these studies combined indicate that *Enterobacter* sp. AA26 might be used as amino acid and vitamin source for both the medfly and the olive fruit fly.

Our study indicates that the use of *K. oxytoca* as a larval diet supplement has negative effects on olive fruit fly rearing. This is contradictory to the results of Kyritsis et al. (2017) in the medfly, where it reduced the immature developmental duration and positively affected adult fight ability. The observed difference is probably due to diverse

dietary needs of the two insect species. *Klebsiella oxytoca* had no significant effect when used as an adult diet supplement in the same medfly study. However, in other medfly studies it affected mating latency time (Ben Ami et al., 2010), male mating competitiveness, female mating receptivity, and longevity (Gavriel et al., 2011). These findings indicate that *K. oxytoca* may produce toxins or other metabolites that are harmful for the olive fruit fly but do not affect *C. capitata*.

The differential response to the same bacterial isolates between *B. oleae* and *C. capitata* might also be attributed to the diverse gut symbiotic communities of the two insect species. It is well-known that the major symbiont of olive fruit fly, *Candidatus* Erwinia dacicola, a Gammaproteobacterium of the Enterobacteriaceae family (Capuzzo et al., 2005), plays a key 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. *Providencia* sp., *Enterobacter* sp., *A. tropicalis*, *Pantoea* sp., *Klebsiella* sp., and *Serratia* sp. have also been identified in lower densities (Sacchetti et al., 2008; Kounatidis et al., 2009; Ben-Yosef et al., 2014; Estes et al., 2014; Koskinioti et al., 2019). However, laboratory adaptation leads to loss of *Ca*. E. dacicola, decrease of bacterial diversity, and gut colonization by other, potentially pathogenic, species such as *M. morganii* (Konstantopoulou et al., 2005; Kounatidis et al., 2009; Estes et al., 2011; Augustinos et al., 2019). On the other hand, *Klebsiella* spp. are mainly found in medfly wild populations and are considered to be significant for *C. capitata* fitness (Behar et al., 2005, 2008; Ben Ami et al., 2010; Gavriel et al., 2011). However, the interaction of *Klebsiella* spp. with the medfly does not appear to be as exclusive as the interaction of *Ca*. E. dacicola with the olive fruit fly. Bacterial diversity also decreases in laboratory-adapted medfly strains, with *Enterobacter* sp. being the most dominant after adaptation (Hamden et al., 2013; Augustinos et al., 2015; Morrow et al., 2015). It is, therefore, obvious that the gut microbe community, the dietary requirements, and the nature of the two fruit fly species are different, which might also explain the different effect of the bacteria additives on their fitness.

The current study is the first that uses gut bacterial isolates as supplements in the larval diet of the olive fruit fly. It is also the first time that a *Bacillus* sp. isolate is used as an additive in tephritid species, which makes the positive effect of *Bacillus* sp. 139 in fruit fly production very interesting, especially because the *Bacillus* genus has never been identified in the natural *B. oleae* gut microbiota before. These findings are a good illustration of the plethora of possibilities that the gut microbiome can provide towards the improvement of insect mass production in support of SIT applications.

In conclusion, the application of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Bacillus* sp. 139, or *Serratia* sp. 49 represents a promising strategy for improvement of the olive fruit fly mass rearing. The beneficial effects of live bacteria can be explained by their ability to colonize the insect gut and provide their host with amino acids, carbohydrates, vitamins, and other nutrients through biosynthesis pathways encoded by their genome. *Providencia* sp. AA31 is a potential candidate for this type of bacteria. However, further research using molecular techniques is needed to confirm the presence of the bacteria in the insect gut after larval feeding. Genome sequencing of *Providencia* sp. AA31 strain further elucidates the gene pathways that contribute to the beneficial effects of the strain. Dead bacteria might provide benefits to their host by (1) direct consumption by the fly larvae, providing amino acids, nitrogen compounds, and other necessary nutrients for larval development, or (2) serving as 'paraprobiotics' that benefit their hosts through non-nutritional pathways by modulating the immune system, increasing adhesion to intestinal cells which inhibits their colonization by pathogens, and by secreting beneficial metabolites by the dead cells. Biochemical and nutritional characterization of these treatments can provide information about the nutrients (amino acids, vitamins, etc.) that these bacteria are able to provide to their host. Some gut bacteria, such as *K. oxytoca*, *Enterobacter* sp. 23, *Providencia* sp., 22 and *Serratia* sp. 49, might be harmful for the insect host and cannot be used as additives in the larval diet. However, some of them such as *Serratia* sp. 49 can be used as a supplement in their dead form. Taking biosafety and biosecurity concerns into consideration, the use of dead/inactivated bacteria is more appropriate for application in real conditions. This would actually exclude the use of *Providencia* sp. AA31 or *Bacillus* sp. 139, but dead *Enterobacter* sp. AA26 is still a promising candidate. More research could further enhance mass rearing by upscaling the experimental design, using more replicates and over more generations, and potentially combining these beneficial isolates or testing new bacteria isolated either from the olive fruit fly or other insect species. Generally, increase of pupal and adult recovery, decrease of the developmental time of the immature stages, and enhancement of longevity of the flies would lead to increased production of insects in shorter time periods. This would facilitate mass rearing of this insect pest species towards not only the efforts for SIT applications, but also for small-scale laboratory rearings required for research purposes.

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Supporting Information

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

Figure S1. Effect of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (+ SEM) adult sex ratio (%; 100× no. males/total no. adults) of the olive fruit fly. No significant difference was observed among the treatments (BLR: $P>0.05$).

Figure S2. Effect of larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Serratia* sp. 49, or *Bacillus* sp. 139 on mean (+ SEM) percentage of fliers in the olive fruit fly. No significant difference was observed among the treatments (BLR: $P>0.05$).

Table S1. Taxonomic identification of the gut bacterial isolates from *Ceratitis capitata* and *Bactrocera oleae* based on the 16S *rRNA* sequencing.

Table S2. Comparison of the gut bacterial isolates belonging to the same genus with pairwise DNA sequence alignments of their 16S *rRNA* sequences.

Table S3. 16S *rRNA* sequences of bacterial isolates known to be Tephritidae gut symbionts retrieved from GenBank.

Table S4. Effect of bacteria-enriched larval diet on the pupal weight (mean ± SEM) of the olive fruit fly.

Table S5. Effect of bacteria-enriched larval diet on the percentage of egg-to-pupa recovery (%; no. pupae/no. eggs) in the olive fruit fly.

Table S6. Effect of bacteria-enriched larval diets on the percentage of egg-to-adult recovery (% number of adults/number of eggs) in the olive fruit fly.

Table S7. Effect of bacteria-enriched larval diets on the percentage of pupa-to-adult recovery (% number of adults/number of pupa) in the olive fruit fly.

Table S8. Effect of bacteria-enriched larval diets on the adult sex ratio (% number of males/total number of adults) of the olive fruit fly.

Table S9. Effect of bacteria-enriched larval diets on the egg-to-adult developmental duration of male and female *B. oleae*.

Table S10. Effect of bacteria-enriched larval diets on male and female adult survival under stress conditions using Cox regression analysis.

Table S11. Pairwise comparisons of the effect of bacteria-enriched larval diets on male and female adult survival under stress conditions using χ^2 test.

Table S12. Effect of bacteria-enriched larval diets on the flight ability of the olive fruit fly using binary logistic regression analysis.