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ORIGINAL ARTICLE

Manipulation of the microbiota of mass-reared Mediterranean fruit flies *Ceratitis capitata* (Diptera: Tephritidae) improves sterile male sexual performance

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The sterile insect technique (SIT) is a method of biological control whereby millions of factory reared sterile male insects are released into the field. This technique is commonly used to combat the Mediterranean fruit fly (*Ceratitis capitata*, Diptera: Tephritidae). Sterile medfly males are less competent in attracting and mating with wild females, a property commonly linked to the irradiation process responsible for the sterilization. As bacteria are important partners in the fly's life cycle, we used molecular analytical methods to study the community structure of the gut microbiota in irradiated male medflies. We find that the sterilizing irradiation procedure affects the gut bacterial community structure of the Mediterranean fruit fly. Although the Enterobacteriaceae family remains the dominant bacterial group present in the gut, the levels of *Klebsiella* species decreases significantly in the days after sterilization. In addition, we detected substantial differences in some bacterial species between the mass rearing strain *Vienna 8* and the wild strain. Most notable among these are the increased levels of the potentially pathogenic species *Pseudomonas* in the industrial strain. Testing the hypothesis that regenerating the original microbiota community could result in enhanced competitiveness of the sterile flies, we found that the addition of the bacterial species *Klebsiella oxytoca* to the postirradiation diet enables colonization of these bacteria in the gut while resulting in decreased levels of the *Pseudomonas* sp. Feeding on diets containing bacteria significantly improved sterile male performance in copulatory tests. Further studies will determine the feasibility of bacterial amelioration in SIT operations.
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Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Diptera: Tephritidae), is a highly polyphagous pest, which infests multiple species of fruits and vegetables worldwide (Liquido *et al.*, 1990). The damage caused by the fly stems from female oviposition in fruits, within which the larvae develop. This development is associated with rapid deterioration of the fruits and vast crop losses. Strategies for area wide integrated management of this invasive pest increasingly use the environmentally friendly approach of the sterile insect technique (SIT) as their central component (Hendrichs *et al.*, 2002). In this technique, male flies are mass reared, sterilized using δ-irradiation and released into the target area where they compete with wild males for copulations with wild females. When a female mates with a sterile male, it has no offspring; thus, the next generation's population is reduced. Various studies have shown that although sterile mass-reared medflies do join mating aggregations ('leks'), they are clearly disadvantaged compared with wild males when competing for wild females (McInnis *et al.*, 1996; Lance *et al.*, 2000; Cayol, 2000; Lux *et al.*, 2002). Consequently, there is a need to improve the competitive ability of the released sterile medfly males to enhance the effectiveness of SIT control programs. Accordingly, various

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approaches have been examined. These include protein supplements to the pre-release diet (Maor *et al.*, 2004; Barry *et al.*, 2007), Juvenile hormone treatments (Teal *et al.*, 2000), probiotic diets (Niyazi *et al.*, 2004), and aromatherapy (Papadopoulos *et al.*, 2001; Shelly *et al.*, 2003). However, with the exception of aromatherapy, none have been implemented on a large scale. This study focuses on the microbiota associated with the digestive system of the sterile fly and the attempt to harness it to improve the insect's mating competitiveness.

Many insects maintain symbiotic relations with bacteria, relations that are associated with a broad spectrum of phenotypes (Bourtzis and Miller, 2003; Rio *et al.*, 2003; Baumann, 2005). Although most of these relations show a bacterial contribution to the metabolism of their host (reviewed by Douglas, 1998, 2009; Bourtzis and Miller, 2003; Dillon and Dillon, 2004), other potential interactions have been suggested. For example, recent studies had shown that the gut microbiota can provide resistance against natural enemies and parasites (Oliver *et al.*, 2003), enhance social interactions (Dillon *et al.*, 2002), and may even assist in various physiological properties that contribute to a longer life span (Behar *et al.*, 2008a). Perhaps the most intriguing aspects regarding gut bacteria–insect associations are how these multi species bacterial communities are structured, the function of each group or species and how these functional groups interact. By combining microbial culture-dependent and cultivation-independent techniques, earlier studies performed by our group have shown that adult medflies harbor a stable and dominant community composed mostly of members of the Enterobacteriaceae family. These are mainly species of *Klebsiella* that are found in different combinations with *Pantoea* spp., *Enterobacter* spp., *Pectobacterium* spp., and *Citrobacter freundii* (Behar *et al.*, 2005, 2008b, 2009). Despite their predominance, the Enterobacteriaecae are not the sole community found in the medfly's digestive system and a stable yet very minor community of *Pseudomonas* spp. bacteria is also present (Behar *et al.*, 2008a). Although the function of these microorganisms is not completely understood, the abundance of pectinolytic and diazotrophic enterobacterial communities (Behar *et al.*, 2005, 2008b) suggests that these bacteria may have significant contributions to fitness during various stages of the host's life cycle (Ben-Yosef *et al.*, 2008a, b).

The effect of the sterilizing irradiation process on mass-reared medflies has been thoroughly studied for quality control reasons with emphasis on parameters such as emergence, longevity, mating competitiveness, and dispersal (Holbrook and Fujimoto, 1970; Zumreoglu *et al.*, 1979; Wong *et al.*, 1982; Barry *et al.*, 2003). However, the effect of irradiation on the gut microbiota has received little scrutiny. As bacteria have significant contributions to various components of fitness (reviewed above), and it is likely that the mass rearing and irradiation processes disrupt the bacteria–fly partnership, we hypothesize that this disruption leads (fully or in part) to the reduced mating competitiveness of sterile males. In light of this hypothesis, in this study we used molecular analytical methods to study the effect of irradiation on the structure of the gut microbiota. We found that, in addition to substantial differences in community structure between the mass rearing strain (*Vienna 8*) and the wild strain, irradiation induced community changes. This led us to test the hypothesis that regenerating the original microbiota could result in enhanced competitiveness of the sterile flies.

Materials and methods

Flies

The mass-reared strain *Vienna 8* flies used in this research were supplied as pupae by Bio-Fly Inc, Sde Eliyahu, Israel. Flies of the 'Sade' strain (that is, the 'lab' strain) were obtained as pupae from the fruit fly facility of the Israeli Citrus Board. This strain is periodically out crossed with males from the wild population, thus preventing the loss of sexual behaviors associated with colonization (Liimatainen *et al.*, 1997). Wild *C. capitata* pupae were obtained from rotting fruits infested with larvae, collected at the Faculty of Agriculture's experimental station (Rehovot, Israel). Pupae and adults were held at 25 °C.

Gut extraction

Flies were dipped in a soap solution, quickly dipped in 70% ethanol for disinfection and washed in sterile saline buffer (PBS). The guts were sterilely extracted by dissection under a microscope and kept in 500 μl of PBS. The guts extract was homogenized using a hand held tissue homogenizer and was used to inoculate growth media for colony analysis or as a sample for DNA extraction (DGGE).

Media, growth conditions, and bacterial strains

Dilution plating of homogenized gut extracts was performed in triplicates, on Luria-Bertani (LB) agar, amended with 500 mg ml^{-1} streptomycin (Sm) when needed, or on *CHROMagar orientation* (HY labs, Rehovot, Israel—Samra *et al.*, 1998) plates followed by incubation for 24 h at 30 °C. *CHROMagar orientation* medium is a chromogenic medium that expedites the identification of various bacteria on the basis of different contrasted colony colors produced by reactions of genus or species-specific enzymes with a proprietary chromogenic substrate. Calibration of the various colors emerging on it was performed by plating different gut isolates previously identified (Supplementary Figure S1). A bias caused by the medium when counting colonies was excluded by counting total culturable bacteria on both the chromogenic medium and on

LB medium. No significant difference was found, with $4.2 \times 10^7 \pm 1.0 \times 10^7$ cfu gut^{-1} counted on the chromo medium compared with $3.7 \times 10^7 \pm 5.0 \times 10^6$ cfu gut^{-1} counted on LB plates (ANOVA: $P < 0.384$, F ratio = 0.9221, d.f. = 1).

A spontaneous Sm-resistant mutant (500 mg ml^{-1} *Klebsiella oxytoca* strain originating from wild-caught flies (Behar *et al.*, 2008a) was isolated on Sm-amended LB plates. The growth rate and final biomass of this strain (thereafter SmKo) in LB broth amended with 500 mg ml^{-1} Sm was not significantly different than those of the wild-type strain grown in non-amended LB (not shown).

Colony analysis

Colonies from the various treatments were randomly selected (150 bacterial colonies from non-irradiated, eclosion day *Vienna 8* flies, 150 colonies from irradiated, eclosion day *Vienna 8* flies, 100 colonies from 5-day-old irradiated *Vienna 8* flies and 100 colonies from field flies), isolated by three cycles of picking and culturing on fresh LB, and had their DNA extracted using the same protocol as mentioned above with the only difference that a single colony was added to the EDTA solution instead of the whole gut. DNA was amplified by polymerase chain reaction (PCR) using the eubacterial 63F-907R (Marchesi *et al.*, 1998; Davidov and Jurkevitch, 2004) and 784F-1401R (Heuer *et al.*, 1997; Behar *et al.*, 2008b) primers sets, targeting the 16S rRNA gene. Amplified rDNA restriction analysis (ARDRA) was performed on these isolates according to Davidov and Jurkevitch (2004). The isolates were grouped according to restriction patterns using the enzymes HaeIII (Takara, Japan), RsaI (Fermentas, USA), and EcoRI (Takara, Japan) and few representatives per group were sequenced for the 16S rRNA gene (Macrogen Inc., Seoul, Korea). Contigs of the two matching PCR products were assembled using DNA Baser v.2.0.3.24 (Heracle Software, Germany).

DNA purification

Total bacterial DNA was extracted from individual fly guts using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) with the following modifications to the manufacture's protocol: Lysosyme (120 µl per sample, 10 mg ml^{-1}) was added to the EDTA solution containing the isolated guts followed by 2 h incubation at 37 °C. Samples were then frozen in liquid nitrogen for 5 min and heated to 65 °C for 100 min. This freezing–thawing process was performed three times in a row. After centrifugation (14 000 *g*, 4 min) and addition of a lysis solution, Proteinase K (17.5 µl per sample, 20 mg ml^{-1}) was added and the samples were incubated for 30 min at 70 °C. The remaining steps were performed as described in the manufacturer's protocol. DNA purification from isolated colonies was performed in the same manner.

Denaturing gradient gel electrophoresis (PCR-DGGE)

To examine the effect of the sterilizing irradiation process on the gut microbial community, total bacterial DNA was directly isolated from dissected guts of non-irradiated *Vienna 8* flies as well as from irradiated ones, on the day of eclosion. Mass produced sterile males are normally reared for five more days in the facility, and are sugar and agar fed, until released into the field. To investigate the possibility that community structure is altered during this period, dissected guts of irradiated 5-day-old flies were also examined. In both cases, the isolated bacterial DNA was subjected to a general bacteria 16S rRNA gene primer's PCR and analyzed with PCR-DGGE. PCR was performed using the eubacterial GC-clamp 968F-1401R primer pair targeting the 16S rRNA gene (Heuer *et al.*, 1997). Amplification was performed with 1 µM of each primer set in 3 mM MgCl, 20 µM of each deoxyribonucleoside triphosphate, 1.25 units of Taq polymerase (Sigma, St Louis, MO, USA) in a total volume of 50 µl of 1 × reaction buffer (Promega). PCR was performed in a PTC-0200 DNA Engine (MJ Research, Waltham, MA, USA) with a denaturation step of 2 min at 94 °C followed by 47 °C for 2 min, 72 °C for 2 min, 94 °C for 1 min, 47 °C for 1 min, 72 °C for 2 min, 23 cycles of 94 °C for 45 s, 47 °C for 45 s, and 72 °C for 45 s and an additional final cycle consisting of 94 °C for 45 s, 47 °C for 45 s, and 5 min at 72 °C for final elongation. PCR products were separated in a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Denaturing gradient gel electrophoresis (DGGE) was performed using a 35–60% urea-formamide gradient (Muyzer and Smalla, 1998) in which each lane represents an individual insect. Gels were stained with SyberGreen and digitally recorded using an Alpha Imager system (Alpha Innotech, San Leandro, CA, USA). Pairwise levels of similarity were calculated using Dice's correlation coefficient and linkage dendograms were constructed from the DGGE gel using the unweighted pair group method with mathematical averages (UPGAMA) algorithm (Discovery Series Quantity one software; Bio-Rad, Hercules, CA, USA). Recurrent (appearing at the same height in the gel) and sporadic bands were extracted from the gels and directly sequenced (Macrogen Inc., Seoul, Korea).

Phylogenetic analysis

16S rDNA sequences obtained from DGGE bands and from bacterial isolates of the ARDRA analysis as well as Enterobacteriaceae 16S rDNA sequences retrieved from the GenBank were added to the rDNA sequence database of the ARB phylogenetic program package (Ludwig *et al.*, 2004). Framework phylogenetic trees were constructed using the ARB's parsimony and maximum-likelihood methods, using a 50% conservation filter. The consistency of the tree was also verified by bootstrapping ($n = 1000$) for the parsimony algorithm.

Experimental diets
The diets included a control, sterile 20% sucrose solution, a diet of live SmKo, and a diet of dead SmKo. The bacteria-containing diets were prepared by spinning down an exponentially growing culture of an Sm-resistance *K. oxytoca* strain and resuspending it to a final concentration of 10^6 cfu ml^{-1} in 20% sterile sucrose. In the dead bacteria diet, the bacterial suspension was autoclaved.

Gut colonization assay
To examine the colonization of the SmKo bacteria in the gut, newly eclosed sterile *Vienna 8* flies were fed bacteria-containing diets for 5 days. The guts of five individuals were dissected at every sampling point (eclosion day, after 3 and 5 days) and its content plated on both 500 mg ml^{-1} Sm-containing LB medium as well as on the commercial chromogenic medium *CHROMagar orientation*. A similar process was performed after two additional days in which the diet was switched to a 20% sucrose-only diet. Colony count on both mediums served as an assay to assess gut colonization.

Copulatory tests
After eclosion flies were housed in 5 L cages, in groups of 100–150 individuals, for 5 days during which three different experimental diets were supplied to four treatment groups: sterile males fed on SmKo enriched diet, sterile males fed the autoclaved SmKo enriched diet, sterile males fed on sucrose only, and lab strain males fed on sucrose only. Flies of the lab strain were separated by gender in the hours posteclosion to ensure that females used in the copulatory tests remained virgins. During the 5 days in which the different diets were supplied, the flies were held at 25 °C and 60% RH. The diet solutions were provided to the flies through cotton wool, and replaced every 24 h. Mating competitiveness tests were conducted under artificial lighting, at 25 °C and 60–65% RH, in 100 L tent cages. Males from the four treatments were separately released into their designated cages 20 min ahead of 3–4-day-old virgin females, allowing them to establish territories. Every cage held 40 males of the same treatment group and 20 females, resulting in an initial male:female ratio of 2:1, respectively. Tests began at 10:00 and lasted for 3 h during which copulating pairs were removed, and latency to copulation was recorded. As the mating system of the medfly is based on female choice, we used latency to mate as a biologically relevant parameter of male quality—the shorter the latency, the greater his perceived value (Yuval and Hendrichs, 2000; Ben-Yosef *et al*., 2008a).

Statistical analysis
Survival analysis was performed to compare the mating latency of the different experimental diets, using the log-rank test. *t*-Test was used to compare the prevalence of the different species in the ARDRA analyses as well as for the colony counts for the colonization by the SmKo strain assays. All the analyses were performed using the JMP7 statistical package.

Results

Analysis of the bacterial community structure in Vienna 8 *flies*
PCR-DGGE analyses revealed that the gut bacterial diversity, as expressed by the total number of bands appearing in the gel, is reduced at eclosion day in the irradiated gut compared with non-irradiated guts and to those of 5-day-old males (3.47 ± 0.22 bands per lane for the irradiated eclosion day gut compared with 5.3 ± 0.39 and 5.55 ± 0.62 bands per lane for the non-irradiated eclosion day gut and 5-day-old gut, respectively) (Figure 1a). These results were confirmed by light intensity profiles calculated for each lane (data not shown). Cluster analysis confirmed that the bacterial community in the irradiated gut was different from the bacterial community in non-irradiated and in 5-day-old flies (Figure 1b). To identify the different communities, recurrently as well as sporadically appearing bands were excised from the DGGE gels and sequenced. A phylogenetic analysis of these sequences revealed that the Enterobacteriaceae family is the dominant bacterial group in the gut, both before and after the irradiation. Although irradiated gut samples from eclosion day contained the genera *Salmonella*, *Citrobacter*, *Providencia*, *Morganella*, and *Enterobacter*, the two other treatments, that is, non-irradiated flies from eclosion days and 5-day-old irradiated flies, contained species of the genera *Klebsiella* and *Pectobacterium* as well. In addition, phylogenetic analyses also revealed that all the *Vienna 8* flies, both before and after irradiation, harbor a stable and dominant community of *Pseudomonas* spp. These species were not detected in any of the wild flies that were used as a control group (Figure 1a).

In addition to DGGE analyses, random colonies obtained from gut extracts by dilution plating on LB medium were isolated and submitted to ARDRA. Representatives from the resulting ARDRA groups were sequenced and identified. The obtained phylogenetic results showed that while the *Klebsiella* spp. is a dominant community among the total gut microbiota of the non-irradiated, 5-day-old irradiated flies and of wild flies (18.67, 23.0, and 31.0%, respectively), its prevalence in the gut of the irradiated flies on eclosion day is significantly lower (4.0%, *t*-test: $t = 2.0129$, $P < 0.05$) (Figures 2a–d). This is in accordance with the absence of *Klebsiella* in identified bands in the DGGE analysis of samples of this latter treatment. ARDRA also confirmed that the gut of the *Vienna 8* flies

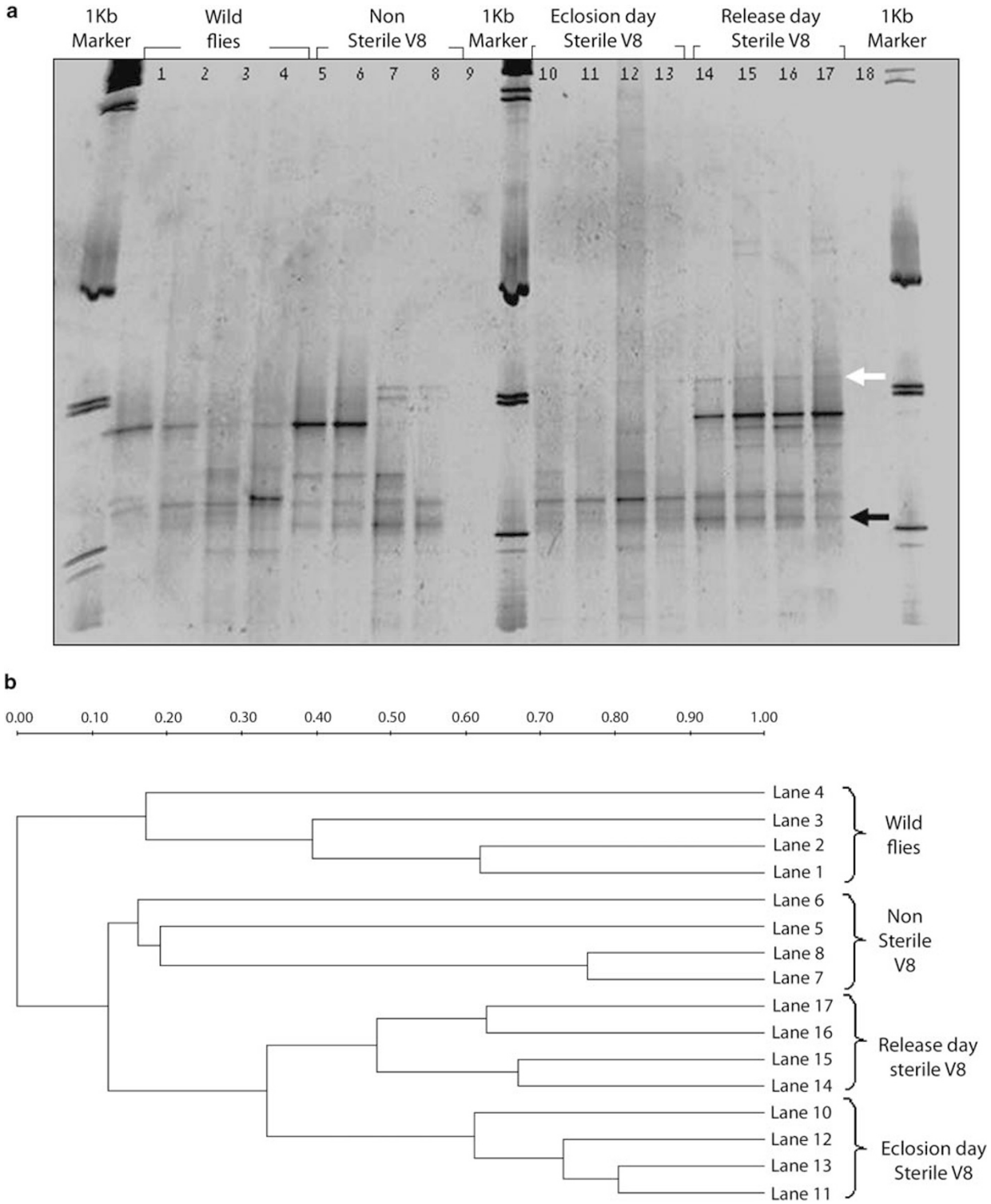

Figure 1 (**a**) Comparative analysis of the microbial community inhabiting the gut of irradiated and non-irradiated flies. Denaturing gradient gel electrophoresis of 16S rDNA gene PCR products obtained from the gut of eclosion day wild flies (lanes 1–4), non-irradiated eclosion day *Vienna 8* flies (lanes 5–8), irradiated eclosion day *Vienna 8* flies (lanes 10–13), and irradiated mass-reared 5-day-old *Vienna 8* flies (lanes 14–17). Lanes 9 and 18 are empty. Products were run in a 35–60% urea-formamide gradient. Bands were excised, sequenced, and subjected to phylogenetic analysis. The black arrow indicates the line of bands identified as *Pseudomonas* spp. Other bands were identified as belonging to the Enterobacteriaceae family as described in the text. (**b**) Dendrograms depicting the relatedness of bacterial communities from the gut of eclosion day wild medfly males, non-irradiated eclosion day *Vienna 8* flies, irradiated eclosion day *Vienna 8* flies, and irradiated mass-reared 5-day-old *Vienna 8* flies. Bacterial community profiles were generated by PCR-DGGE analysis shown in (**a**).

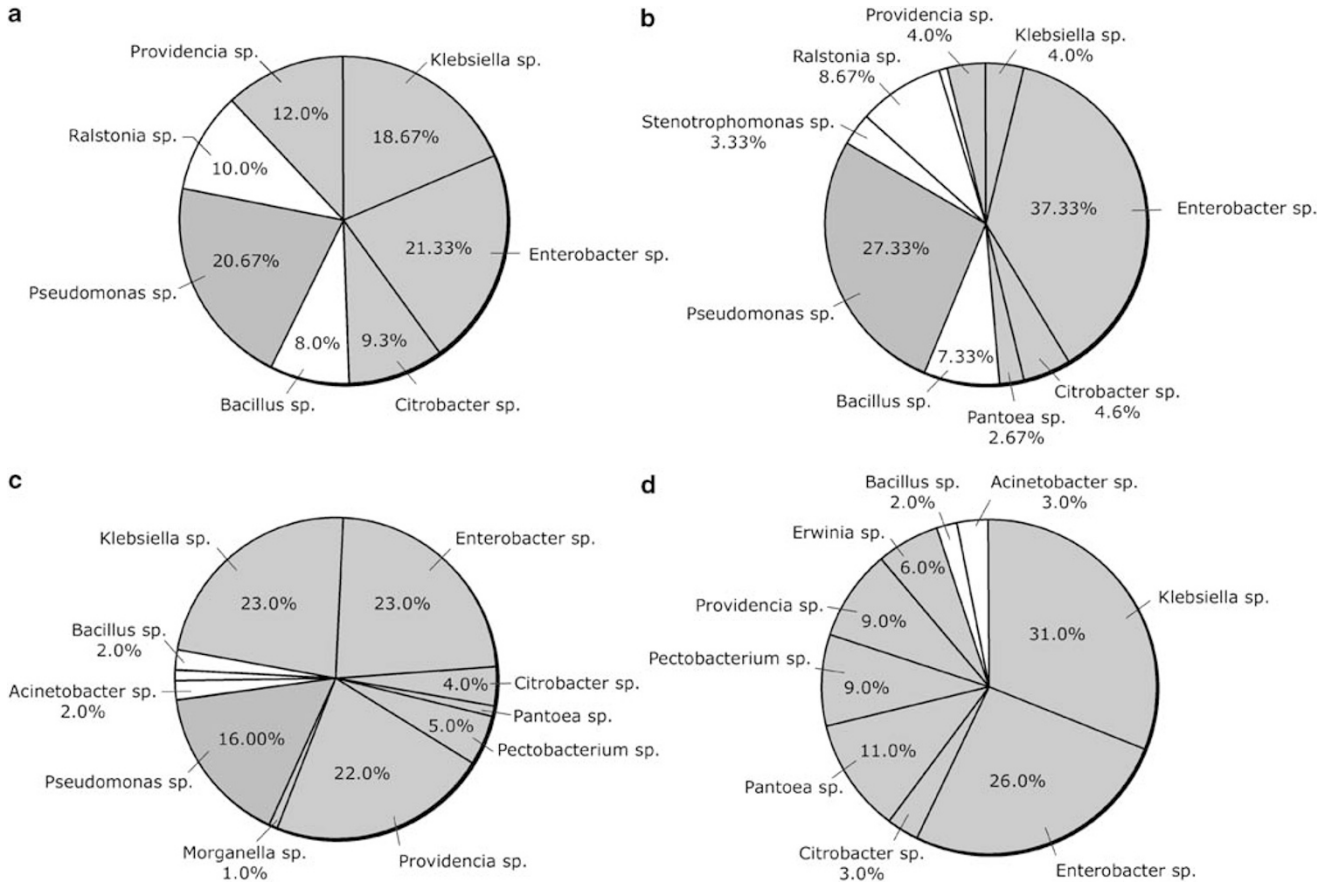

Figure 2 Pie charts representing the microbial gut community of (**a**) non-irradiated eclosion day *Vienna 8* flies, (**b**) irradiated eclosion day *Vienna 8* flies, (**c**) irradiated mass-reared 5-day-old *Vienna 8* flies, and (**d**) eclosion day wild flies. Blue slices indicate communities belonging to the Enterobacteriaceae family. Red slices indicate communities identified as *Pseudomonas* species. Colonies isolated from guts were grouped by ARDRA. The 16S rRNA gene of group representatives was sequenced and their phylogenetic affiliation determined using the ARB package. A full colour version of this figure is available at *The ISME Journal* online.

harbors a dominant population of *Pseudomonas* spp. that is not detected in the gut of wild flies (Figures 2a–d).

Colonization of the sterile male gut by the SmKo strain
Sterile male flies were fed a sucrose diet enriched with a concentrated suspension (10^{-6} cfu ml^{-1} of the SmKo strain for 5 days. We then examined the bacterial content of the gut by plating it on both antibiotic containing LB medium and on the commercial chromogenic medium *CHROMagar orientation*. Total bacterial counts on LB plates significantly increased throughout these 5 days as well as during the following 2 days in which the diet was switched to a sucrose-only diet ($P<0.0025$, $F=18.7167$, d.f. $=1$) (Figure 3a). As expected, after 5 days, the total number of colonies in the SmKo-amended diet group was significantly higher compared with the sucrose-only diet control group ($P<0.0021$, $F=19.9502$, $DF=1$). Plating the guts content on the chromogenic agar yielded colonies of three different colors: blue (as *K. oxytoca*), yellow (as *Pseudomonas* spp.), and white-beige (as *Providencia* and *Morganella* spp.) (Supplementary Figures S1 and S2). The number of blue colonies grew significantly throughout the 5 days in which the SmKo-amended diet was supplied as well as in the following 2 days in which the diet was switched to sucrose only, and was significantly higher compared with the sucrose-only control group ($P<0.0018$, $F=20.8748$, d.f. $=1$) (Figure 3b). Random sampling of blue colonies ($n=10$) who were subjected to 16SrRNA gene sequencing, identified them as belonging to the genera *Klebsiella* and *Enterobacter*. Finally, plating gut extracts on Sm-LB plates showed that although no resistant colonies were detected before the ingestion of the SmKo-containing diet, they were observed after 3 and 5 days (Figure 3d). Replica-plating onto to the chromogenic medium only yielded blue colonies. A random sample of these colonies ($n=10$) was subjected to 16SrRNA gene sequencing, identifying them all as belonging to the species *K. oxytoca*. Although the number of blue, Sm-resistant colonies increased, the number of yellow colonies decreased gradually throughout the 5 days of the SmKo-amended diet (Figure 3c). They could not be

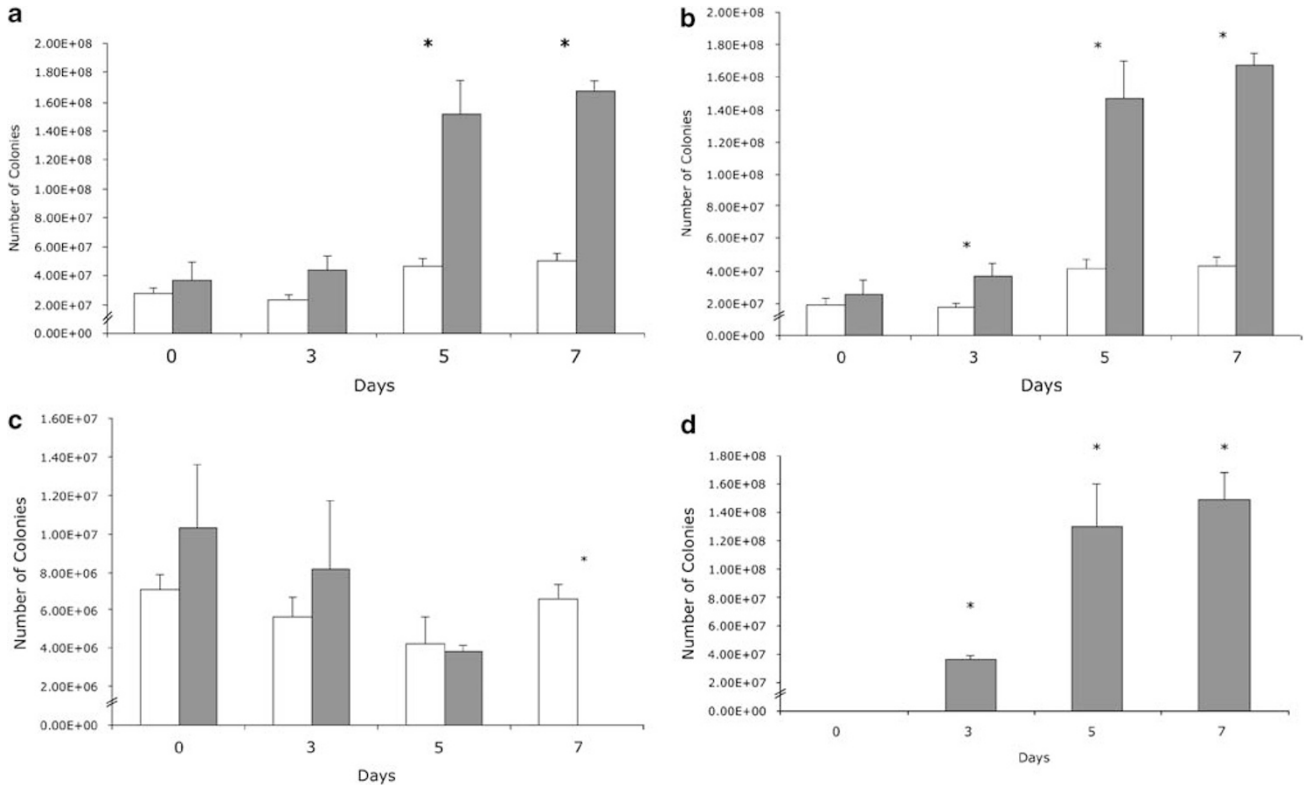

Figure 3 Colonization of the sterile male gut by the Sm-resistant *Klebsiella oxytoca* SmKo strain. Number of bacterial colonies retrieved from the guts of medflies fed with a diet containing the SmKo strain, growing on a *CHROMagar orientation* chromogenic medium, during a 7-day-long experiment. (**a**) Total colonies, (**b**) blue colonies, (**c**) yellow colonies. (**d**) Number of Sm-resistant colonies growing on a Sm-containing LB medium (identified by 16SrRNA sequencing as *Klebsiella oxytoca*). White bars represent the control group that were fed on sucrose-only diets whereas the dark bars represent flies fed Sm-resistant *Klebsiella oxytoca*.

detected during the next 2 days after the flies were switched to a sucrose-only diet. In comparison, no resistant colonies were detected throughout the entire 7 days in the control group that was fed with the sucrose-only diet, and the number of yellow colonies did not change significantly. Random sampling of yellow colonies ($n=5$) and 16SrRNA gene sequencing identified them all as belonging to the genus *Pseudomonas*. White-beige colonies were found at much lower level than the blue or the yellow ones. However, they also decreased in the SmKo-amended treatment (data not shown). Random sampling ($n=3$) and 16SrRNA gene sequencing identified them as belonging to the genera *Providencia* and *Morganella*.

Effects of the SmKo strain on copulatory success of sterile males

To assess the effect of the SmKo-enhanced diet on the copulatory success of the sterile males, we conducted parallel copulation tests for sterile male flies that were fed the diet containing bacteria for 5 days compared with three control groups (Figure 4). Copulatory tests showed that feeding on the SmKo-amended diet significantly shortened the mating latency of the sterile males. Males fed on this diet started mating significantly faster than sterile males fed only on sucrose (log-rank, DF $=1$, $\chi^2=16.973$, $P<0.0001$) and lab strain males also only fed on sucrose (log-rank, d.f. $=1$, $\chi^2=4.665$, $P<0.0308$). To exclude the possibility that the behavioral effect stemmed from the added bacteria serving as a nutritional source, a treatment of autoclaved SmKo cells added diet was also examined. Compared with males that fed the autoclaved bacteria, the sterile males fed live bacteria averaged significantly shorter mating latencies (log-rank, d.f. $=1$, $\chi^2=28.145$, $P<0.0001$).

Discussion

The sterilizing irradiation procedure affects the gut bacterial community structure of the Mediterranean fruit fly. In this study, we have shown that although the Enterobacteriaceae family remained the dominant bacterial group present in the gut, the proportion of *Klebsiella* spp., the most dominant species in field flies (Behar *et al.*, 2005) decreased significantly after sterilization. In addition, we were able to show that substantial differences exist in the composition of the gut community between the mass-reared strain *Vienna 8* and the wild strain. Most notable

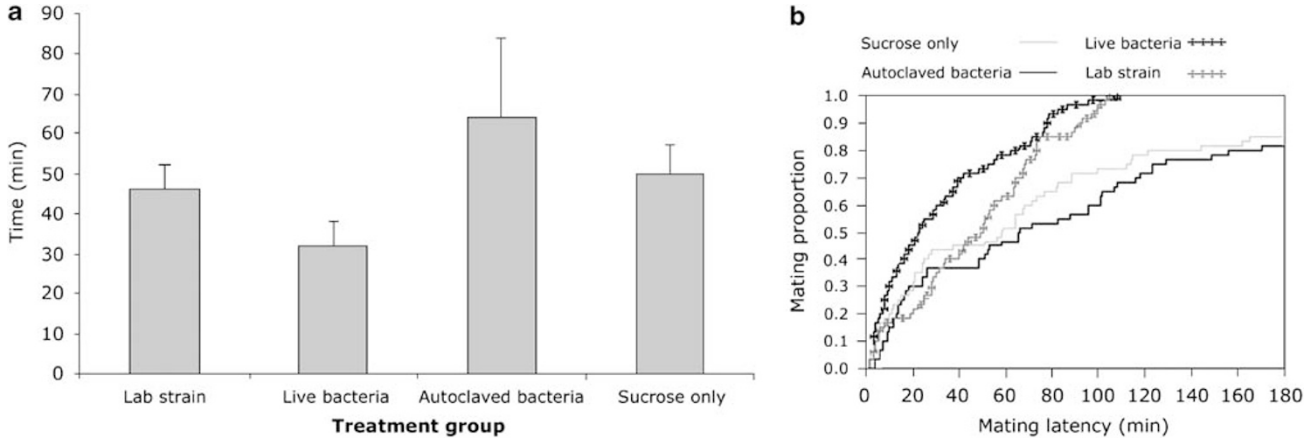

Figure 4 Effects of *Klebsiella oxytoca* SmKo-amended diet on mating latency. Average (**a**) and plot (**b**) of the mating latency of sterile medfly males fed live *Klebsiella oxytoca* SmKo cells, autoclaved SmKo cells, or sucrose only. The mating latency of a lab strain medfly's males who were fed sucrose was also measured.

among these are the increased levels of the potentially pathogenic genus *Pseudomonas* in the industrial strain. Testing the hypothesis that regenerating the original microbiota community could result in enhanced competitiveness of the sterile flies, we found that the addition of the bacterial species *K. oxytoca* to the postirradiation diet enables colonization of these bacteria in the gut while resulting in decreased levels of the *Pseudomonas* spp. Copulatory success tests show that the addition of these bacteria to male diets significantly improved sterile male performance.

Our culture-dependent and independent analyses of the bacterial community inhabiting the gut of the medfly's *Vienna 8* strain revealed that despite their dominance, Enterobacteriaceae are not the sole bacterial community in the gut. Bacteria of the genus *Pseudomonas* constitute a common and stable community as well. These findings are interesting when considering that earlier data have shown that in the wild strain, these bacteria constitute only a very minor and rare community of about 0.005% that could not be detected in standard PCR-DGGE procedures (Behar *et al.*, 2008a). These findings could also be significant for the SIT as various *Pseudomonas* species are known to harm different insects: *P. entomophila* is a known pathogen of *Drosophila* flies (Vodovar *et al.*, 2005), *P. fluorescens* was found to be lethal to mosquitoes and to the house fly *Musca domestica* (Padmanabhan *et al.*, 2005) and *P. aeruginosa* is a known pathogen of the nematode *C. elegans*, of *Drosophila* and of the larvae of *Hylesia metabus* (Osborn *et al.*, 2002; Apidianakis *et al.*, 2005; Hilbi *et al.*, 2007). Furthermore, providing low, but higher than natural, levels of *Pseudomonas aeruginosa* to wild medflies results in significant reduction of longevity (Behar *et al.*, 2008a). These results suggest that at least part of the *Pseudomonas* community present in the gut can cause damage to its medfly host when occurring at, or reaching, higher densities than normally found. Our finding that the *Vienna 8* strain contains a massive community of *Pseudomonas* suggests that these microorganisms may have a negative effect on the competitiveness of the mass-reared fly.

In addition, we found that newly eclosed sterile males contained low levels of *Klebsiella* spp. in their gut. They were completely absent from the DGGE analyses of eclosion day sterile flies and were rarely found on plates. *Klebsiella* species are of high prevalence in the wild medfly's gut, constituting the most common species (Behar *et al.*, 2005, 2008a). Although the functional role of these bacteria in the gut is only partially understood, there is evidence that *Klebsiella* spp. performs nitrogen fixation *in vivo*, in the fly's gut (Behar *et al.*, 2005), and that the bacteria are associated with male mating success (Ben-Yosef *et al.*, 2008a). These results, as well as those presented in this study, and the fact that nitrogen is a limiting factor in the reproductive success of both female and male Mediterranean fruit flies in nature (Yuval *et al.*, 1998; Yuval and Hendrichs, 2000) suggest that the decreased levels of the *Klebsiella* after the irradiation results in lower levels of available nitrogen to the fly. We were able to show that the addition of *K. oxytoca* to a liquid diet presented to the sterile flies leads to a significant increase in the levels of these bacteria in the gut. In addition, we showed that as the *Klebsiella* levels rise, the levels of *Pseudomonas* decrease. When taking into account the potentially harmful effect of *Pseudomonas*, one could postulate that the bacteria-enhanced diet could improve the fly's performance. Our copulatory success tests show that the *K. oxytoca*-enhanced diets significantly shortened the mating latency of the sterile males. As the number of sexually receptive females in the field is generally low, and the most attractive males are selected first, latency provides a reliable measure of ecological realism. Accordingly, we are

encouraged to suggest that bacteria-enhanced diets may be implemented to improve sterile male performance.

In conclusion, this study presents evidence that the SIT's sterilizing irradiation process affects the microbial community structure in the medfly's gut. In addition, we find that the mass-reared *Vienna 8* strain exhibits a modified bacterial profile compared with wild flies and that these modifications, combined with the irradiation effects, might play a significant role in the low competitiveness of sterile males. The improvement in the mating latency of sterile males fed *K. oxytoca* has encouraging potential for improvement of the SIT.

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