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Source: Journal of Economic Entomology, 114(2) : 560-570

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jee/toaa294

Journal of Economic Entomology, 114(2), 2021, 560–570
doi: 10.1093/jee/toaa294
Advance Access Publication Date: 18 January 2021
Research

Manipulation of Gut Symbionts for Improving the Sterile Insect Technique: Quality Parameters of *Bactrocera dorsalis* (Diptera: Tephritidae) Genetic Sexing Strain Males After Feeding on Bacteria-Enriched Diets

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Subject Editor: Nikolaos Papadopoulos

Received 8 February 2020; Editorial decision 22 November 2020

Abstract

One environmentally friendly method used to manage *Bactrocera dorsalis* (Hendel), a key agricultural pest of substantial economic importance, is the sterile insect technique (SIT). Nevertheless, several deficiencies related to this strategy impair the success of the SIT, including the inferior performance of released sterile males compared with wild males, which could be partly solved by the utilization of gut symbionts as probiotic dietary components. In this study, a culture-dependent method was used to isolate and characterize gut-associated bacterial species in adult *B. dorsalis* genetic sexing strain (GSS) males. In addition, three bacterial isolates from the Enterobacteriaceae family, namely, *Enterobacter* sp., *Morganella morganii*, and *Moellerella wisconsensis*, were used as supplements in larval and adult diets to assess their effects on the life-history traits of irradiated males. Consistent with many previous studies, *Enterobacter* spp. was shown to be beneficial, with some quality control indices, such as adult size, pupal weight, survival rate under stress and nutritionally rich conditions, and mating competitiveness, being significantly increased, while slight nonsignificant increases in emergence rate and flight ability were observed. Conversely, the *M. morganii* and *M. wisconsensis* strains both had negative effects on irradiated male fitness and mating competitiveness. Our results, in combination with those of earlier studies, can contribute to improving the effectiveness of SIT application by enhancing the different aspects of augmentative rearing and biological traits of pests under laboratory rearing conditions.

Key words: oriental fruit fly, GSS, irradiation, SIT, bacteria-enriched diet

As an active component of area-wide integrated pest management (AW-IPM), the sterile insect technique (SIT) has been widely employed to combat various tephritid pests, including *Anastrepha ludens* (Loew) (Sánchez-Rosario et al. 2017), *Ceratitis capitata* (Wiedemann) (Silva et al. 2013), *Bactrocera dorsalis* (Hendel) (Shelly et al. 2010) and *Zeugodacus cucurbitae* (Coquillett) (Ito et al. 2003) (Diptera: Tephritidae). The SIT refers to the augmentative rearing and release of sterile insects that induced by irradiation targeting natural populations of the same species, resulting in sterile crosses and subsequent population suppression (Knipling 1955). The successful application of an SIT programme heavily depends on the feasibility of continuously supplying an adequate number of high-biological-quality insects (Calkins and Parker 2005). In most SIT programmes, insect sterilization is accomplished through irradiation (Cai et al. 2018a). Some previous reports have revealed that sterile males show inferior fitness and sexual performance compared with those in wild populations (Rull et al. 2005, Ben-Ami et al. 2010). To obtain optimal control effects, the mass-rearing production (quantity) and outdoor performance (quality) of released sterile flies should be as excellent as or even stronger than those of targeted species (Dyck et al. 2005). The factors influencing the quantity and quality of mass-cultured flies may include colonization habitat, the environmental rearing conditions, the irradiation process and prerelease and release operations (Knipling 1955, Rull et al. 2005). Furthermore, the poor fitness and field performance of sterile males caused by irradiation may be associated with injured tissues or internal organs and with

the condition of the symbiotic community of their digestive system (Lauzon et al. 2012).

Recent works combining microbial culture-independent and microbial culture-dependent methods have shown that stable and abundant symbiotic communities can colonize Tephritidae species, such as *B. dorsalis* (Wang et al. 2011, 2014; Gujjar et al. 2017), *Z. cucurbitae* (Gujjar et al. 2017), *B. minax* (Rashid et al. 2018), *B. oleae* (Ben-Yosef et al. 2014), *B. zonata* (Saunders) (Naaz et al. 2016), *B. papayae* (Drew and Hancock) (Thaochan et al. 2014), *B. neohumeralis* (Hardy), *B. jarvisi* (Tryon), *B. cacuminata* (Hering) (Morrow et al. 2015), *B. carambolae* (Drew &Hancock) (Yong et al. 2017), *B. tryoni* (Froggatt) (Thaochan et al. 2010), *Rhagoletis pomonella* (Walsh) (Lauzon et al. 2003), *A. ludens* (Kuzina et al. 2001), *Zeugodacus tau* (Walker) (Prabhakar et al. 2013, Luo et al. 2018) and *C. capitata* (Behar et al. 2008b, Ben-Ami et al. 2010, Kyritsis et al. 2017). These reports demonstrated that the symbiotic communities consisted mainly of the genera *Klebsiella*, *Enterobacter*, *Pectobacterium*, *Pantoea*, *Providencia*, *Citrobacter* and *Morganella*, which belong to members of the Enterobacteriaceae family. These bacteria that occur within the intestines of flies have crucial functions in hosts development and reproduction (Liu et al. 2018) and significantly contribute to host fitness at various stages (Ben-Yosef et al. 2008), affecting host gut physiology, tissue homeostasis, and environmental stress tolerance and enhancing the availability of nutrient resources and host resistance to pesticides and pathogens (Montllor et al. 2002, Engel et al. 2013, Broderick et al. 2014, Cheng et al. 2017).

By using the SIT, encouraging findings have been documented for the application of different bacteria as probiotic supplements in larval or/and adult diets to solve the quality problems that may be caused by disrupting the gut symbiota during mass rearing and and/or sterile irradiation (Ben-Ami et al. 2010, Gavriel et al. 2011, Lauzon and Potter, 2012, Hamden et al. 2013). A number of studies have clarified that the fitness and mating competitiveness of released sterile males used in the SIT are significantly increased after feeding on gut-associated bacteria compared with non-bacteria fed flies (Gavriel et al. 2011, Hamden et al. 2013, Kyritsis et al. 2017). For example, a cultivable bacterial species (*Enterobacter* sp.) was isolated from the alimentary tracts of *C. capitata* genetic sexing strain (GSS) males (VIENNA 8D53+) and added to the larval diet, resulting in faster development of immatures and reduced mortality during the immature stage (Augustinos et al. 2015).

Other authors extended this research by evaluating the probiotic effects of another gut symbiont, *Klebsiella oxytoca*, on VIENNA 8D53+ adults by assessing different biological quality parameters (Ben-Ami et al. 2010). Their findings indicated that (a) sterile males fed with the bacteria were more effective in preventing female receptivity than only sugar-fed males and that (b) feeding sterile males with *K. oxytoca* significantly increased their mating success, shortened their mating latency time and improved their ability to survive under food deprivation. It follows that the incorporation of bacteria into the diet of sterile males is a valid method to enhance their fitness and enable them to be more effective in an SIT system. Outside of *C. capitata*, probiotic diets have only been studied in only a few other tephritid pests, such as *Z. tau* (Khan et al. 2014), *B. oleae* (Sacchetti et al. 2014), *Z. cucurbitae* (Yao et al. 2017), *B. tryoni* (Meats et al. 2009), *B. dorsalis* (Khaeso et al. 2018, Khan et al. 2019) and *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae) (Rull et al. 2015).

Bactrocera dorsalis, commonly named the oriental fruit fly, is a pest of economic importance that poses serious threats to numerous fruits and vegetables (Cai et al. 2017). The extensively studied biology of *B. dorsalis* makes it an ideal tephritid model for AW-IPM applications, including the SIT approach. For oriental fruit flies, a significant improvement in SIT application has been the exploitation and utilization of GSSs, which conditionally produce only males (Mccombs et al. 1995, Ji et al. 2007a). SIT applications in many tephritid pests have demonstrated that releasing only males was more efficient and cost-efficient than bisexual releases (Franz et al. 2005). Studies assessing the use of *B. dorsalis* in SIT programmes have mainly focused on their genetics, their copulating behavior, field applications, and optimization of irradiation processes rather than on the application of probiotic additives to improve biological quality (Ji et al. 2007, Ji et al. 2013, Augustinos et al. 2014, Zahan et al. 2016).

Recently, Zhang et al. (2018) and Cai et al. (2018c) both showed that irradiation destroyed the microbial balance in the intestinal tract of *B. dorsalis* GSS males, decreasing the abundance of functional and specific gut bacteria of the Enterobacteriaceae family, which was closely associated with decreased ecological fitness including male mating competitiveness, flight ability, survival rate and longevity in this tephritid fly (Cai et al., 2018c). Moreover, Cai (2018c) and Khan et al. (2019) indicated that gut symbiont-based probiotics can be utilized as agents for restoring the declined ecological fitness induced by irradiation, which can be used in conjunction with SIT. Within the above framework, the aims of the present study were to 1) isolate and characterize the bacterial species in the gut of *B. dorsalis* GSS males and 2) compare the effects of incorporating *Enterobacter* sp., *M. morganii* and *M. wisconsensis* into the diet on the quality parameters of laboratory-reared *B. dorsalis* GSS males exposed to irradiation.

Materials and Methods

Fly Colonies

The *B. dorsalis* GSS strain was reared in accordance with the procedures proposed by Ji et al. (2007) and maintained for almost 60 generations at the Institute of Beneficial Insects, Fujian Agriculture and Forestry University, Fujian, P.R. China (BII, FAFU). The laboratory-reared *B. dorsalis* GSS female pupae were white, whereas the male pupae were brown (Ji et al. 2007). The wild-type fly colonies used in the mating competitiveness experiments were reared from larvae obtained from rotten citrus fruit initially collected from Fuzhou city, Fujian Province, P.R. China, and maintained at BII, FAFU. All adults were reared in cages with fine mesh on two sides and were provided with water and food containing yeast hydrolysate and sugar at a weight ratio of 1:3. These rearing cages (30×30×30 cm) were held under laboratory conditions at 25 ± 1°C, 65 ± 5% relative humidity (RH) and a photoperiod of 14:10 (L:D).

Isolation and Identification of Gut Bacteria

Guts were collected from 12-d-old nonirradiated *B. dorsalis* GSS adult males. Before dissection, all individuals were subjected to 3–5 min of cuticle-sterilization using 70% ethanol and washed thoroughly in sterile deionized water three times. Thirty adult males were aseptically dissected using sterile tweezers in a Petri dish that contained 1 ml of sterile distilled water under a stereomicroscope (Olympus SZX12, Tokyo, Japan). After dissection, the collected guts were transferred into 1.5-ml tubes containing 1 ml of sterile phosphate-buffered saline solution and homogenized. The homogenate was serially diluted to 10^{-6} and spread onto Luria–Bertani (hereafter referred to as LB) agar medium. Three bacterial suspension concentrations of 10^{-4}, 10^{-5}, and 10^{-6} were chosen as samples and spread onto the LB agar medium and cultured at 37°C for 24–48 h. All sample treatments were replicated three times to ensure

that bacterial growth could be inspected in a timely and correct manner. Well-isolated colonies were selected from all sample treatments. Multiple streaking and isolation operations were conducted to ensure that the isolates represented a single colony. Over 50 colonies were chosen for further analysis on the basis of colony morphology and ensuring that representatives from all sample treatments were included.

Single isolates were transferred from the LB solid agar medium to 1-ml microfuge tubes containing LB liquid medium using a sterile inoculating loop under sterile conditions. Sequentially, these tubes were deposited in an incubator shaker at a constant temperature of 37°C to culture the bacteria at 200 revolutions per minute for 48 h. To amplify the bacterial 16S rRNA gene segment, polymerase chain reaction (PCR) amplification was conducted from the bacterial solutions using the universal bacterial primers 27F/1492R (Edwards et al. 1989, Weisburg et al. 1991). The total PCR volume was 25 µl, containing 18 µl of ddH_2O, 2.5 µl of 10× PCR buffer I, 0.5 µl of 29× Trans Taq HiFi PCR SuperMix, 0.5 µl of 10 mM each primer, 2.0 µl of 10 mM dNTPs, and 1 µl of template DNA. The PCR was conducted under the following conditions: an initial denaturing step of 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 sec, and annealing at 56°C for 30 sec, and extension at 72°C for 60 sec. A final extension step of 72°C for 5 min was added. Three microlitres of each reaction product was electrophoresed on a 1.5% agarose 1× Tris–Borate–EDTA gel. Negative controls without the addition of DNA were routinely used to check for contamination. Good-quality PCR products were sent to Beijing Genomics Institute (BGI, Shenzhen, China) for sequencing with an ABI 3730 XL using the aforementioned primers. The 16S rRNA gene sequence from each isolate was compared and aligned to available sequences in the GenBank database through the BLAST search algorithm (Altschul et al. 1997). The completed and matched sequences were applied to establish a phylogenetic tree using neighbor-joining (NJ) algorithms in MEGA 5.0 (Tamura et al. 2011), and the accuracy of the tree topology was assessed by 1000 bootstrap replicates (Felsenstein et al. 1985, Thompson et al. 1997).

Preparation of the Bacteria-Enriched Diet and the Sterilization Process

The larval diet consisted of yeast hydrolysate as a protein source, wheat bran as a bulking source, sugar as a phagostimulant and carbohydrate source and water, and the adult diet was composed of sugar and whole yeast (3:1 ratio; Spencer and Fujita, 1997). The three tested bacterial species, namely, *Enterobacter* sp., *M. morganii* and *M. wisconsensis*, were isolated from the guts of nonirradiated *B. dorsalis* GSS males aged 12 d old. For each bacterial species, 600 ul of bacterial suspension was added to a 200-ml shaker flask containing 150 ml of autoclave-sterilized LB liquid medium. The shaker flasks were deposited in a constant-temperature incubated rotary shaker at 200 revolutions per min for 96 h at 37°C. Before adding the bacteria, all larval and adult diets (including those of the control group) were autoclaved at 121°C for 20 min and then cooled to room temperature. Forty-five milliliters of LB liquid medium containing the bacteria was used to prepare one thousand grams of larval diet. For the adults, 15 ml of the LB liquid medium containing the bacteria was prepared in a flask to produce four hundred grams of diet. To eliminate the possible effects of nutrients contained in the bacterial growth medium, an equal volume of autoclave-sterilized medium excluding any bacterial species was added to the control diet. Sterile males were acquired by exposing pupae to gamma irradiation from a ^{137}Cs source at 100 Gy two days prior to emergence (Ji et al. 2007). Four diet types were tested: no bacteria (control group, CK), *Enterobacter* sp.-enriched diet (E), *M. morganii*-enriched diet (MM) and *M. wisconsensis*-enriched diet (MW).

Quality Parameter Determination

The quality parameters of the flies reared from different bacterial-enriched diets and the control were determined by evaluating pupal weight (mg), pupal mortality (%), emergence rate (%), flight ability (%), survival under stress (%), mating competitiveness, longevity (d) and morphological indices. All quality parameters were evaluated under controlled laboratory conditions (25 ± 1°C, 65 ± 5% RH).

Pupal Weight

One milliliter of *B. dorsalis* eggs was respectively seeded onto the four types of larval diets and maintained on a plate (50×20×4 cm) that was deposited into closed boxes (80×40×20 cm) with wet sand to allow pupation. Pupal weight was evaluated by individually weighting 100 male pupae per treatment two days prior to emergence through a semimicro balance (CP225D, accuracy of 0.01mg, Sartorius, Göttingen, Germany; FAO/IAEA/USDA 2014).

Pupal Mortality, Emergence Rate, and Flight Ability

To assess the effects of the bacteria-enriched diet on the pupal mortality, emergence rate and flight ability of *B. dorsalis* males, brown pupae from each treatment were deposited in a black plastic cylinder (diameter: 10 cm, height: 20 cm) for emergence. Talcum powder was daubed on the inner surface of each tube containing 100 pupae to prevent the flies from walking out the tube. All plastic cylinders were deposited in large test cages (1.2 × 1.2 × 1.2 m) that were held under the insectary conditions mentioned above. The numbers of deformed flies (A), normal flies that did not fly out of the cylinder (B), half-emerged pupae (C), and dead pupae (D) were counted four days after set up. The pupal mortality (M%), emergence percentage (E%), and flight capability (F%) were calculated by the following formulas: M% = D; E% = 100 – C – D; F% = 100 – A – B – C – D (FAO/IAEA/USDA 2014). Nine replicates of each treatment were performed.

Survival Rates Under Stress Conditions

In the following experiments, only adult males without perceptible deformation were tested. The effects of a bacteria-enriched larval diet on the adult male survival rate under illumination, food and water deprivation conditions (stress conditions) were assessed. Within 4 h of adult emergence (08:00–12:00 am), 50 irradiated males per treatment were deposited in large Petri dishes (100 × 20 mm) with an opening of approximately 15 mm covered with mesh and lacking diet and water. All dishes were deposited in the dark at 25 ± 1°C and an RH of 65 ± 5%. After 48 h, live and dead flies were quantified to determine the percent survival (FAO/IAEA/USDA 2014). Five replicates were conducted per treatment.

Mating Competitiveness

At the early stage, *B. dorsalis* GSS males were assigned to one of the following larval diets (hereafter referred to as LD): LD/LB (control), LD/*Enterobacter* sp., LD/*M. morganii*, LD/*M. wisconsensis*. The wild fly larvae were fed a nonbacterial larval diet. Wild flies were sexed immediately after emergence (within 4 h) and continuously fed with a sugar/protein-based adult diet (hereafter referred to as AD) without any bacteria and respectively maintained in

small cages (30×30×30 cm). Two days before emergence, *B. dorsalis* GSS brown pupae (males) were sterilized by ^{137}Cs radiation according to the protocol mentioned above. After emergence, irradiated *B. dorsalis* GSS males reared on each larval diet with any of the supplements were continuously provided the corresponding adult diet. For example, *B. dorsalis* GSS males were fed LD/LB at their larval stage and then fed AD/LB at their adult stage. For all treatments, the adult diet was offered ad libitum and replaced daily.

One day prior to the experiment, the thoraxes of wild and treated males were marked with spots of different-coloured spots of a nontoxic dyes (either red or yellow) which did not influence mating performance (Kaspi and Yuval, 2000a). The color was alternated between replicates to remove bias. Mating assays were performed in cages (60×60×60 cm) under the conditions described above. The *B. dorsalis* GSS males and the wild flies (females and males) used in this experiment were all 11 d old. On the testing day, 50 wild females and 50 males of each of the two groups (wild males and irradiated males fed AD/LB [control], AD/*Enterobacter* sp., AD/*M. morganii* or AD/*M. wisconsensis*) were isolated in the testing cages. Subsequently, these cages were visually checked every 10 min until no copulation occurred. Mating couples were gently transferred to other small cages, and the number of pairs per male type (fed or not fed with bacteria and GSS or wild males) was counted. The relative index (RI) is analogous to the relative sterility index (FAO/IAEA/USDA, 2014) and was calculated as the number of bacteria-treated males that mated with wild females divided by the total number of mating actions that occurred (for both treated and control males). Five replicates for each treatment were performed.

Longevity

The rearing process of *B. dorsalis* GSS males at the larval and adult stages was in accordance with the procedure previously described in the assessment of mating competitiveness. To estimate longevity, one group of 50 irradiated male flies reared on their respective larval diet were immediately placed in the corresponding small cages (30 × 30 × 30 cm) after emergence. A piece of filter paper was used to soak the fermented bacteria solution. The adult food and fermented bacteria solution were provided throughout the testing and renewed daily. For example, the irradiated *B. dorsalis* GSS males fed LD/LB during larval stage were offered AD/LB during their adult stage. Male mortality was recorded daily until all males died. Five replicate experiments were conducted.

Morphological Parameters

The effects of the bacteria-enriched larval diet on the size of the irradiated *B. dorsalis* GSS adult males were evaluated using morphological indices, including head width (HW), abdomen length (AL), wing length (WL), and thorax length (TL), based on the protocol of Yao et al. (2017). Samples of 100 males fed with and without bacteria were evaluated under a stereomicroscope fitted with Motic Images Plus software v.2 (Rodriguero et al. 2002).

Statistical Analysis

Statistical analysis was carried out using SPSS. 17.0 (SPSS, Inc., Chicago, IL, United States). The data for pupal weight (g), pupal mortality, emergence rate (%), flight ability (%), and mating competitiveness were analyzed by one-way ANOVA and Tukey's honestly significant difference test, whereas the survival rates were analyzed with the log-rank test. To improve normality and homoscedasticity (Zar et al. 1984), all percentage data were arcsine square root transformed, but untransformed values are exhibited in the figures.

Results

Gut Bacterial Diversity of the *B. dorsalis* GSS Males

The bacterial colonies isolated from the 12-d-old nonirradiated *B. dorsalis* GSS male guts were morphologically similar, indicating the existence of a low-complexity community of bacteria that are aerobically culturable and can grow on LB agar. Out of the 60 colonies from the guts of 12-d-old nonirradiated *B. dorsalis* GSS males subjected to 16S rRNA gene sequencing, 20 were identified as *Citrobacter* sp., 19 as *Enterobacter* sp., 8 as *Escherichia* sp., 2 as *Pantoea* sp., 3 as *Morganella* sp., 2 as *Moellerella* sp., 1 as *Klebsiella* sp., 1 as *Providencia* sp., 1 as *Enterococcus* sp., 2 as *Lactococcus* sp. and 1 as *Bacterium* sp., which could be assigned to the Enterobacteriaceae and Streptococcaceae families (Table 1). Overall, sequences from *Enterobacter* sp., *Escherichia* sp., *Citrobacter* sp., *Pantoea* sp., *Morganella* sp., *Moellerella* sp., *Klebsiella* sp., *Providencia* sp., *Enterococcus* sp., *Lactococcus* sp., and an unknown bacterial species accounted for 31.66, 13.33, 33.33, 3.33, 5.00, 3.33, 1.66, 1.66, 1.66, 3.33, and 1.66% of all the examined sequences, and Enterobacteriaceae dominated within the gut of *B. dorsalis* GSS males under the culture conditions used in the study. The phylogenetic tree analysis according to partial sequencing of the 16S rRNA gene (at least 1,000 bp, 500 bp from each end) further demonstrated the presence of a bacterial gut community of low complexity (Fig. 1).

Pupal Weight

The bacteria-enriched larval diet exerted significant effect on *B. dorsalis* GSS male pupal weight ($F_{3,36}$ = 15.48, df = 3, P < 0.001). The mean male individual pupal weight significantly increased by 0.2736 mg after the addition of *Enterobacter* sp. to the larval diet compared with the weights of those fed without bacteria (control group). However, small, nonsignificant decreases in male pupal weight were recorded with the diets containing *M. morganii* and *M. wisconsensis* added compared with the control group (Fig. 2).

Pupal Mortality

There were significant differences in the pupal mortality among the different bacterial treatments ($F_{3,16}$ = 7.92, P < 0.001). Adding *M. morganii* or *M. wisconsensis* to the larval diet significantly

Table 1. Bacterial communities isolated from the adult gut of *B. dorsalis* GSS male aged at 12 d old

	Isolated bacteria										
	Enterobacteriaceae								Streptococcaceae		Unknown
Genus	*Enterobacter*	*Escherichia*	*Citrobacter*	*Pantoea*	*Morganella*	*Moellerella*	*Klebsiella*	*Providencia*	*Enterococcus*	*Lactococcus*	*Bacterium*
Colony no.	19	8	20	2	3	2	1	1	1	2	1

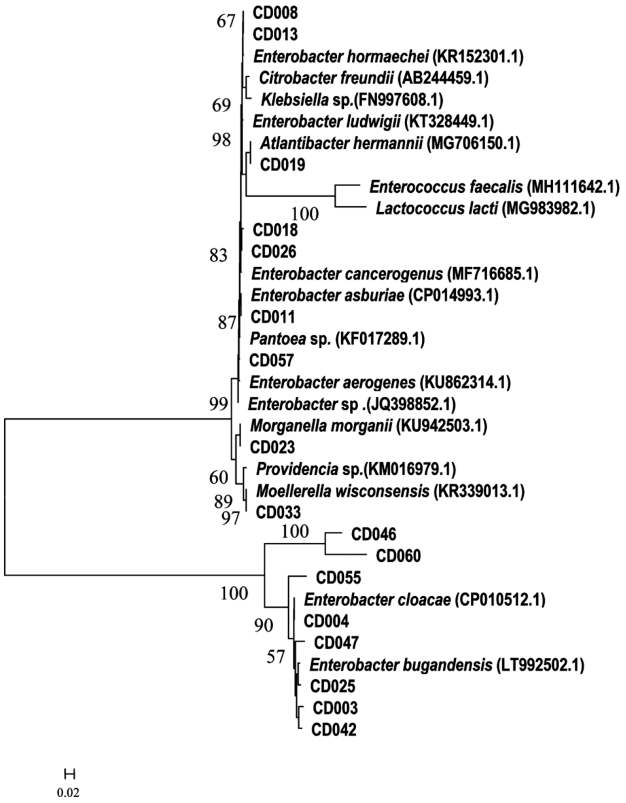

Fig. 1. Dendrogram based on 16S rRNA sequences. *Enterobacter* sp., *Citrobacter* sp., and *Escherichia* sp. were isolated from the guts of nonirradiated *B. dorsalis* GSS males. Analysis was conducted using MEGA 5.0 software. The phylogenetic tree was inferred using the neighbor-joining method and drawn to scale, with branch lengths in the same units as those of the evolutionary distances applied to generate the tree.

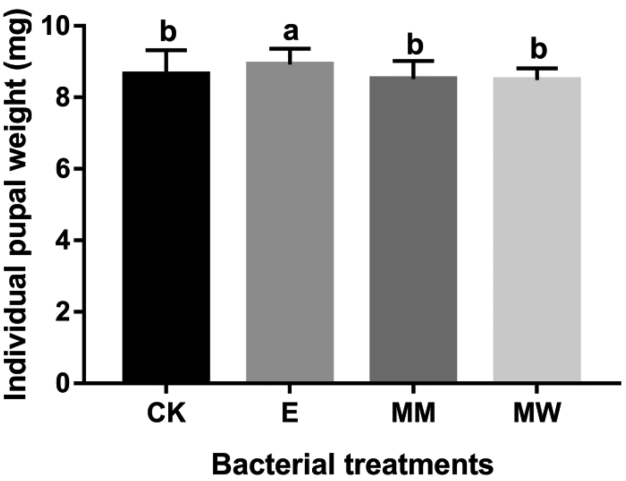

Fig. 2. Pupal weight (mean ± SE) of the irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval diets. Bars topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

increased the male pupal mortality, while a mild non significant reduction was observed in the *Enterobacter* sp. treatment group in comparison with the control group (Fig. 3).

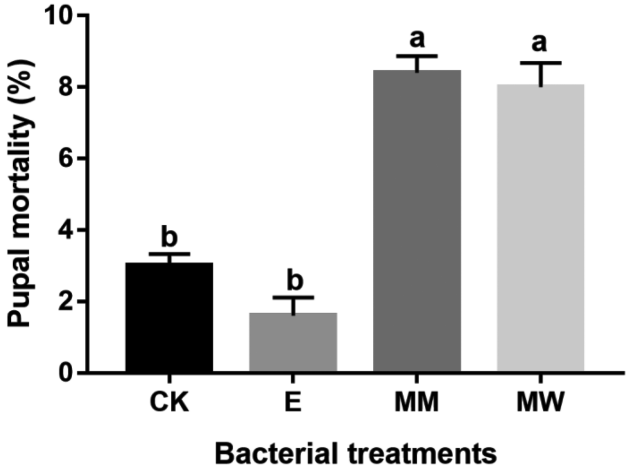

Fig. 3. Pupal mortality (mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval diets. Bars topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

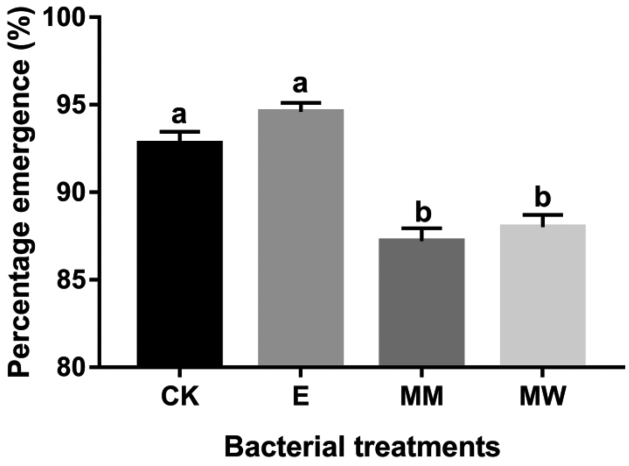

Fig. 4. Emergence percentage (mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval diets. Bars topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

Emergence Percentage and Flight Ability

There were significant differences in the emergence percentage and flight ability among the different bacterial treatments (emergence percentage: $F_{3,16} = 30.00$, $P < 0.001$; flight ability: $F_{3,16} = 23.05$, $P < 0.001$). The mean percentages of emergence were significantly reduced after incorporating *M. morganii* or *M. wisconsensis* into the larval diet, whereas the addition of *Enterobacter* sp. to the larval diet slightly improved the emergence of irradiated *B. dorsalis* GSS males, but this difference was not significant compared with the control group (Fig. 4). The results of the flight ability test were similar to the results of emergence percentage (Fig. 5).

Survival Under Stress Conditions

A significant difference was observed in survival percentages among the different bacterial treatments ($\chi^2 = 36.07$, df = 3,16, $P < 0.001$).

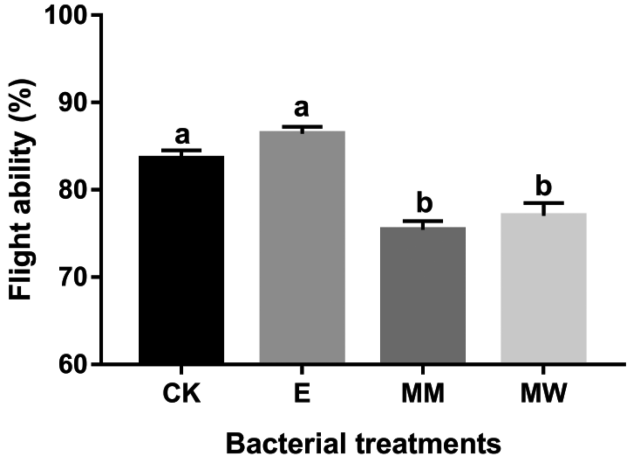

Fig. 5. Flight ability (mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval diets. Bars topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

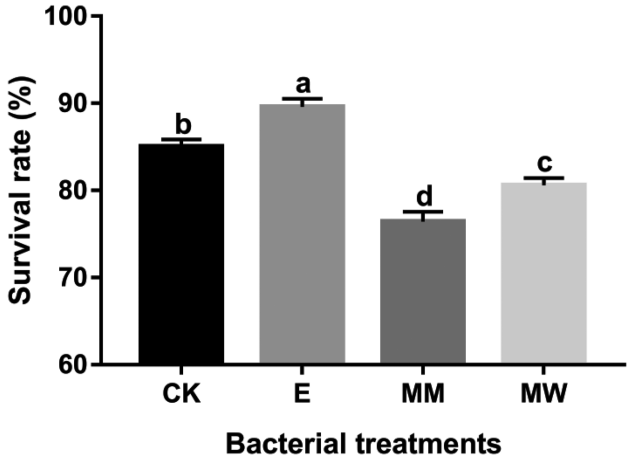

Fig. 6. Survival rates (Mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval diets under stress conditions within 48 h. Bar topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

Incorporating *Enterobacter* sp. into the larval diet led to a significantly higher survival rate than that of the control group, which was contrary to the effect of the *M. morganii* or *M. wisconsensis*-enriched diets (Fig. 6).

Mating Competitiveness

The incorporation of *Enterobacter* sp. into the larval and adult diets had a significantly positive effect on irradiated males' sexual competitiveness for wild females. Based on the comparison of the RI parameter, the mating competitiveness of the irradiated males reared on each of the bacterial treatments except *Enterobacter* sp. did not significantly differ, but the competitive ability of the irradiated males from these two bacterial treatments was significantly

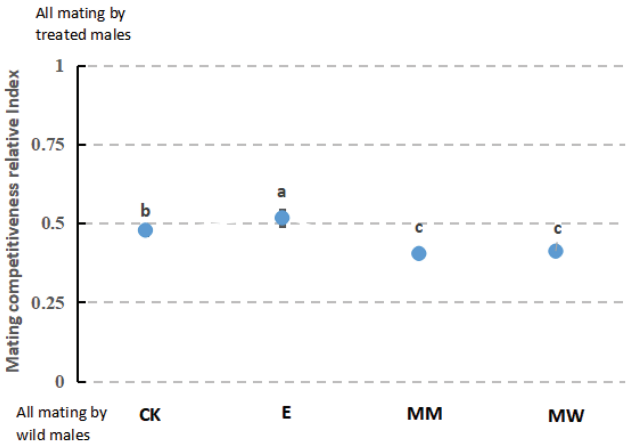

Fig. 7. Mating competitiveness (mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched diets. Points topped with the same letter do not significantly differ (Tukey's honestly significant difference test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

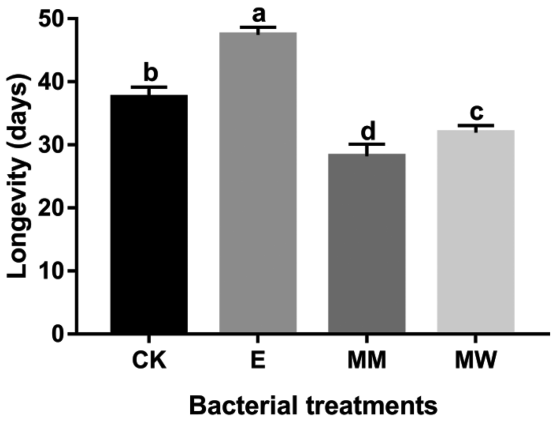

Fig. 8. Mean longevity (mean ± SE) of irradiated *B. dorsalis* GSS males reared with bacteria-enriched larval and adult diets under nutritionally rich conditions (log-rank test, $P > 0.05$). CK, control group; E, *Enterobacter* sp. supplementation treatment, MM, *M. morganii* supplementation treatment; MW, *M. wisconsensis* supplementation treatment.

lower than that of the males in the control group ($F_{3,16} = 23.22$, $P < 0.001$, Fig. 7).

Longevity

The irradiated males that were fed *Enterobacter* sp.-enriched larval and adult diets lived significantly longer than the males that were not fed diets containing bacteria, and the irradiated males had a significantly shorter life when they were fed a *M. morganii* or *M. wisconsensis*-enriched diets ($\chi^2 = 9.72$, df = 3,16, $P < 0.001$, Fig. 8).

Morphological Parameters

For all morphometric indices, the irradiated males that were fed a larval diet containing *M. morganii* or *M. wisconsensis* were significantly smaller than the males fed the control diet with no bacteria. Conversely, the irradiated males that were fed the *Enterobacter* sp.-enriched larval diet were significantly larger than the males in the control group, with a significantly larger HW ($F_{3,396} = 6.27$,

Table 2. Effects of bacterial provision diet on the morphometric traits of irradiated *B. dorsalis* GSS males under stress conditions.

Parameter	Bacterial treatment			
	CK (Without bacteria)	*Enterobacter* spp.	*M. morganii*	*M. wisconsensis*
Head width (mm)	1.80 ± 0.02b	1.88 ± 0.04a	1.69 ± 0.01d	1.73 ± 0.02c
Thorax length (mm)	2.52 ± 0.01b	2.60 ± 0.06a	2.34 ± 0.03d	2.43 ± 0.01c
Abdomen length (mm)	2.73 ± 0.01b	2.80 ± 0.02a	2.64 ± 0.03c	2.66 ± 0.03c
Wing length (mm)	5.05 ± 0.03b	5.22 ± 0.01a	4.84 ± 0.02c	4.86 ± 0.03c

Means (±SE) within row followed by the same letter do not differ significantly (Tukey's honestly significant difference test, $P > 0.05$).

$P < 0.001$), AL ($F_{3,396} = 3.25$, $P < 0.001$), TL ($F_{3,396} = 3.22$, $P < 0.001$), and WL ($F_{3,396} = 5.38$, $P < 0.001$, Table 2).

Discussion

The analysis of the isolated bacteria indicated that members of the Enterobacteriaceae family were dominant in the alimentary canals of nonirradiated *B. dorsalis* adult males, which is consistent with previous studies showing a prevailing Enterobacteriaceae community in other tephritid pests of the genera *Bactrocera* (Jones et al. 2013), *Ceratitis* (Behar et al. 2008a,b), and *Anastrepha* (Kuzina et al. 2001). However, minor differences between the results of the present study and those of previous studies in terms of the microbial diversity in the digestive systems of various fly populations occurred due to differences in the fly species, strains, and developmental stages/ages tested, diets, and experimental conditions. The wide distribution of Enterobacteriaceae in the fly gut communities revealed that this family likely plays a supportive role in host metabolism. Many Enterobacteriaceae are diazotrophic bacteria that can readily metabolize nitrogenous compounds for their hosts (Behar et al. 2005). Furthermore, the enterobacterial communities in the intestinal tracts of *C. capitata* could be beneficial to host fitness by preventing the proliferation or establishment of pathogenic bacteria (Behar et al. 2008a,b). In addition, many previous studies have suggested that the inoculation of diets with *Enterobacter* spp. confers beneficial effects on recovery rates and the developmental duration of immatures in *C. capitata* (Augustinos et al. 2015) and significantly enhances some quality control (QC) parameters, including pupal weight, survival rate under stress conditions and adult size in *Z. cucurbitae* (Yao et al. 2017).

Kyritsis et al. (2017) suggested that the similar (or even the same) bacterial taxa do not consistently play a probiotic role and that certain nutrients provided by bacteria may be crucial in some insect foods but not in others. The probiotic effects of the supplied bacteria vary under different rearing conditions and in association with different diet components and fly species. Thus, evaluation of the effects of the enrichment of diets with different bacterial species from the Enterobacteriaceae family in other tephritid pests is warranted. Our objective was to screen cultivable bacterial species suitable for probiotic applications in diets used in mass-rearing programmes. The effects of these bacteria were tested with regard to the rearing productivity and biological traits of *B. dorsalis* GSSs based on the standard QC parameters applied in SIT applications and mass-rearing facilities (FAO/IAEA/USDA 2014). Moreover, we focused on assessing the effects on males because they are the active component in SIT operational systems (Yao et al. 2017).

A previous study reported that enriching the larval diet with *Enterococcus phoeniculicola* could be beneficial in increasing the pupal weight and survival percentage and in shortening the larval developmental duration. However, diets enriched with *Lactobacillus lactis* could play a pathogenic role in *B. dorsalis* development (Khaeso et al. 2018). The findings of our research can be summarized as follows: 1) the diet-based probiotic application of *Enterobacter* sp. as 'live bacteria' or their byproducts led to significantly enhanced pupal weight, adult size, survival rate under stress and nutritionally rich conditions and mating competitiveness. 2) Feeding on a diet containing this bacterial strain tended to decrease pupal mortality and increase the emergence rate and adult flight capability, although the differences between the bacterial treatment and control groups were not statistically significant. 3) Supplementation with *M. morganii* or *M. wisconsensis* did not result in any advantage regarding the aforementioned QC parameters and had adverse effects on irradiated *B. dorsalis* GSS males. Thus, our study confirms that gut bacteria should be regarded as one of the most important factors affecting the development of the associated host flies (Khaeso et al. 2018).

Many reports have revealed that irradiation has a negative effect on sexual behavior (Lux et al. 2002), life-history traits (Barry et al. 2003), antioxidant enzyme activities (Cai et al. 2018a) and other gut digestive enzymes of insects (Boshra et al. 2007). Lauzon and Potter (2012) revealed that structural injury is consistent with the impact of ionizing irradiation and results in physiologic and metabolic abnormalities and consequences that significantly weaken the competitiveness of fruit male flies. Our findings after supplementing probiotics into the larval diet corroborate those of several previous studies (Niyazi et al. 2004, Gavriel et al. 2011) that assumed that the dietary supplementation with probiotics could ameliorate the adverse effects of irradiation on gut bacterial communities. Analogous effects on ultrastructure, histology and physiological functions were found in rats (Demirer et al. 2006). In this research, the addition of *Enterobacter* sp. to the larval diet at the larval stage still resulted in pronounced improvement in the same QC parameters after adult emergence, even among the males that were fed diets supplemented with bacteria and that had received irradiation. This result is plausible because microorganisms can be vertically transmitted during all developmental stages of *C. capitata* from larvae to adults (Behar et al. 2008b, Lauzon et al. 2009). Compared with the males that were not fed diets supplemented with bacteria, the relative abundance of the gut bacterial communities in the males fed the bacteria-enriched diets likely increased to some extent, which led to the enhanced quality and energetic mating activities observed in our study.

Furthermore, the significant enhancement of the QC parameters observed in the irradiated *B. dorsalis* GSS males after feeding on *Enterobacter* sp.-enriched diets could be explained by the hypothesis of Behar et al. (2005), who attributed the easy availability of metabolizable nitrogen and carbohydrates in attacked fruits to the nitrogen fixation and pectinolytic processes of the enterobacterial community in the intestinal tracts of *C. capitata*. We speculated that the same activities occurred in the larval diet enriched with *Enterobacter* sp., providing high levels of available nutritional resources for the larval development and increasing the larval capacity for food ingestion.

This process would equip larvae with a better capability to accumulate nutritional resources, produce heavier pupae, improve adult emergence, longevity and flight capability and generate larger and healthier adults. Furthermore, offering digestive enzymes or vitamins and/or by increasing digestion efficiency would eventually contribute to the development and survival of the hosts (Ben-Yosef et al. 2014, Nash and Chapman, 2014).

Notably, these positive effects are of major importance in augmentative rearing and in the large-scale application of the SIT. For instance, released irradiated males that have been fed bacteria-enriched diets have more vitality regardless of whether they experience food- or water-deficient or nutritionally abundant conditions and have more powerful flight and sexual competitive ability; these traits are responsible for reducing the costs of SIT systems and improving their success (Kyritsis et al. 2017). In addition, heavy pupal weight is considered an anticipated trait in fruit flies reared on artificial diets, leading to the production of males with greater competitiveness. Acquisition of a higher pupal weight has been documented to be influenced by the amino acid content in diets and may be attributed to the symbiotic bacteria that help hosts to extend their nutritional range (Wong et al. 2014). Nestel et al. (2004) suggested that pupal weight is influenced by the amino acid content in diets. Our findings showed that adding *Enterobacter* sp. to larval diets may have triggered some behavioral or physiological changes that caused a corresponding improvement in pupal weight. Numerous studies have correlated size and copulatory success, that is male size may be a factor affecting female mating receptivity, and larger adult males have greater reproductive success than smaller ones (Kaspi et al. 2000b, Hamden et al. 2013).

The bacterial community harbored in the insect gut is not always beneficial to flies (Khaeso et al. 2018). For example, the addition of the naturally occurring gut symbionts *Microbacterium* sp., *Klebsiella pneumoniae*, *Vagococcus fluvialis*, *Lactococcus lactis*, and *M. morganii* to larval diets significantly prolonged the larval developmental duration of *B. dorsalis* GSSs. Moreover, inoculation with *L. lactis* was the most harmful because it significantly prolonged fly development and decreased the total number of surviving flies (Khaeso et al. 2018). In our study, we also assessed the effects of two naturally occurring gut symbionts in mass-reared *B. dorsalis*, namely, *M. morganii* and *M. wisconsensis*, the abundances of which have been demonstrated to increase in the gut after receiving the radiation (Zhang et al. 2018). The results showed a significant alterations in the pupal morality, emergence rate, flight ability, survival rate under both conditions and mating competitiveness in irradiated males fed diets containing these two bacteria as well as a small and nonsignificant decrease in pupal weight. Survival may be influenced by nutrient absorbance during the larval phase (Nash et al. 2014). From this perspective, the ingestion of these two gut bacteria damages larvae and adults as a result of feeding or inhibits them from maximizing the absorbance of nutrients from the consumed diet, which may partially explain the detrimental effects on all QC parameters.

In a study by Khaeso et al. (2018), *M. morganii* was not seen as a detrimental bacterium; although feeding on this bacterium could significantly extend the larval developmental duration, the total number of eggs that survived to the adult stage was not significantly decreased compared with the number in the control treatment. Notably, *M. morganii* has been recorded as a cause of accidental food contamination and different types of animal diseases, such as meningitis, peritonitis, bacteremia, and urinary tract and wound infection. Thus, this bacterium has been categorized as an opportunistic pathogen that poses a threat to the aquaculture industry and human health and has led to some reported deaths in recent years (Shahbazi et al. 2019). In addition, *M. wisconsensis* seems to be widely distributed in nature and has been associated with peritonitis, diarrhea and acute cholecystitis on rare occasions. Several strains have been isolated from water, animals, human food and clinical specimens, such as stools (Stock et al. 2003). Previous reports have suggested that *M. morganii* is an extremely lethal pathogen of *A. ludens* larvae (Salas et al. 2017), but little information is available for other fruit fly species. Their negative roles of these bacteria in fruit flies remain unclear, suggesting the need for extra studies in this area. From the viewpoint of improving SIT effectiveness, ways to reduce harmful bacteria and increase beneficial bacteria within the diet are needed to strengthen the fitness of released SIT males.

Although the use of diets with probiotic supplementation in mass-rearing facilities and SIT are promising and encouraging, it is far from becoming the default approach. Even if live bacteria may seem to be more effective than 'dead' bacteria in remedial actions based on previous reports since the nutritive value of the killed bacterial cells is probably negligible in a diet that is already abundant in nutrients. However, supplementation with autoclaved bacteria is more realistic due to the convenience and biosecurity of the process of storage and usage (Kyritsis et al. 2017, Yao et al. 2017). Further studies should test the practical effects of probiotic supplementation on mass-rearing techniques for SIT applications by exploiting effective and widely accepted manipulation procedures. This can be done by investigating the effects of the Enterobacteriaceae family members derived from wild fly guts on sterile fly behavior and physiology to identify a more nutritionally complete diet to increase laboratory-reared fly fitness, assessing the effectiveness of different probiotic bacteria individually or in combination, and conducting a cost–benefit analysis of the industrial production of probiotics for the mass rearing of sterile flies. In our research, bacteria were not enumerated in the diets during or after larval development, making it difficult to conclude if manipulations actually resulted in their establishment in the diet. Nevertheless, treatments resulted in significant effects, suggesting that bacteria remained active in the diets/larval or adult guts. In the near future, some quantitative molecular techniques, such as quantitative PCR could be employed to enumerate individual species as well as total bacteria using species-specific probes and can detect a broad linear range from 10 to more than 10^8 cells (Raza et al. 2020).

In conclusion, our results further confirm that the addition of probiotics to diets mitigates irradiation-induced effects on the intestinal bacterial communities in flies. This approach could be integrated in mass-rearing facilities worldwide and in SIT application after the optimization of the various parameters, such as the dose, application frequency, and species of the bacterial strains to be incorporated into the larval/adult diets to acquire mass-released sterile males with good quality. This process could be used in conjunction with the recently recommended reduction in irradiation doses (Cai et al. 2018b) to obtain a good balance among the quality, competitiveness, and sterility of fruit flies in SIT operational programmes.

Acknowledgments

We express our thanks to Mingyan Yao and Mijuan Luo for their technical assistance. We thank the Crop and Nuclear Technology Utilization Institute at Zhejiang Academy of Agriculture Science, Hangzhou, Zhejiang, P.R. China, for providing the irradiation and operation guidance. This research was funded by the National Key R&D Program of China (2017YFD0202100, 2017YFD0201008, 2017YFD0202000),

Industry University Research Project of Fujian Science and Technology Department (2019N5003), the Advanced Talents Introduction Project of Wuyi University (YJ201910), Science & Technology Innovation Platform Construction Project of Fujian Province (2018N2004) and Education and Scientific Research Project for Young and Middle-aged Teachers in Fujian Province (JAT190801, JAT190805).

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