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Diversity of the cultivable gut bacterial communities associated with the fruit flies *Bactrocera dorsalis* and *Bactrocera cucurbitae* (Diptera: Tephritidae)

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Abstract Gut microbes play an important role in insect morphogenesis, nutrition, development of resistance against parasitoids and detoxification of toxic compounds. A culture-based approach is therefore an useful tool for the characterization of cultivable microbial communities associated with the insect gut. In the present study an attempt was made to decipher the gender specificity of gut bacterial communities of two major fruit fly species of India viz., *Bactrocera dorsalis* (Hendel) and *Bactrocera cucurbitae* (Conquillett) (Diptera: Tephritidae). Based on molecular identification, *B. dorsalis* females were found to predominantly harbor the bacterial species *Enterobacter cloacae*, *Enterobacter asburiae* and *Citrobacter freundii*, while *B. dorsalis* males were found to harbor *Providencia rettgerii*, *Klebsiella oxytoca*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* The cultivable diversity from females of *B. cucurbitae* comprised mainly of *Morganella morganii* and *Bacillus pumilis* while *B.cucurbitae* males were predominantly colonized by aerobic endospore formers viz., *Bacillus cereus*, *B. licheniformis* and *B. subtilis*. The above findings have thrown light on a distinct pattern of gender specific gut bacterial colonization in fruit flies, which have to be factored in for the formulation of fruit fly management strategies.

Keywords Gut bacteria · *Bactrocera dorsalis* · *Bactrocera cucurbitae* · Diversity

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Introduction

Tephritid flies commonly known as fruit flies and are serious pests of various fruit and vegetable crops throughout the world (Gullan and Cranston 2010). The fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera:Tephritidae) is polyphagous pest that infests more than 250 host plants, including commercial fruit crops the most important being mango (*Mangifera indica*) (Verghese et al. 2012). Similarly the melon fly, *Bactrocera cucurbitae* (Coquillett), is a major pest of vegetables. It has been recorded on more than 125 species of plants, including cucurbits and tomatoes (Weems et al. 2015). It is well known that arthropods are associated with large and diverse microbial communities which reside in their digestive system (Dillon and Dillon 2004). The associations of insects with microbes has a long history of co-evolution which ranges from parasitism to obligate symbiosis (Dale and Moran 2006). Microbial symbionts play

a significant role in the biology and evolution of many insect groups (Baumann 2005). The bacteria associated with insects biosynthesize essential amino acids (Miyazaki et al. 1968) and sometimes serve as food sources (Drew and Lloyd 1989). Other functions include the conversion of undigestable food components into forms that are easily digested by the insect (Lauzon et al. 2000), detoxification of allelochemicals present in food and atmospheric nitrogen fixation (Behar et al. 2005). Gut microbes also influence insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds (Genta et al. 2006).

The association of bacteria with fruit flies has been well known (Petri 1910), and certain bacterial species are known to form intimate, symbiotic associations with tephritids (Marchini et al. 2002). Tephritid flies harbour different bacterial symbionts in their digestive system, which influence different fitness parameters (Pramanik et al. 2014).

Several bacterial species belonging to family Enterobacteriaceae viz., *Klebsiella oxytoca, Enterobacter cloacae*, *Citrobacter freundii,* and *Providencia rettgeri* were isolated from the alimentary tracts of four *Bactrocera* species *(*Lloyd et al. 1986). Several species of bacteria were also isolated from fly feces, host fruit surfaces, oviposition sites and larvae infested fruit tissues. But the gender specific association of cultivable bacterial diversity amongst *Bactrocera* species has not been well established so far, therefore the main objective of this study was to isolate and characterize the cultivable gut bacteria associated with the male and females of *B. dorsalis* and *B. cucurbitae* using a combination of morphological, physiological, biochemical and 16S rRNA gene sequence based analysis.

Materials and methods

Raising of the stock culture

Bactrocera dorsalis and *B. cucurbitae* cultures were established using field collected larvae that infested mango and cucumber fruits respectively. The larvae were reared on the natural hosts in cages (40 cmx40 cmx45 cm) at room temperatures (25 ± 2 °C) in the Fruit Entomology Laboratory of the Division of Entomology and Nematology, ICAR-Indian Institute of Horticultural Research (12° 8′N; 77° 35′E), Bengaluru, India. A tray filled with a 5 cm layer of fine sand was kept inside the cage for pupation. The pupae were carefully collected and kept in a separate cage for adult emergence. The adult feed was supplemented with sugar, *B*-protein, yeast extract and water soaked in cotton swabs. Gravid females of *B. dorsalis* adults were exposed to banana fruits (cv. *Elakki*) for 24 h for oviposition. The banana fruits where oviposition occurred were kept in plastic containers partially filled with fine sand and covered with a muslin cloth, for larval development. The last instar maggots were then transferred to a plastic container with sand for pupation, in cages (Jayanthi and Verghese 2001). Ripe pumpkin fruits were used for rearing of *B. cucurbitae* by following the procedure described above. Five generations of *B. dorsalis* and *B. cucurbitae* were reared and maintained in the laboratory and used for the study.

Isolation of gut bacteria

A total of five, 12 days old laboratory reared male and gravid female flies of each species were separated and cold anesthetized at −20 °C for 5 min, surface sterilized with 70% (*v*/v) alcohol for 60 s, followed by another round of sterilization for 60 s using 0.5% sodium hypochlorite (*v*/v). The surface sterilized fruit flies were washed thoroughly with sterile distilled water thrice and aliquots of the last wash water were plated on nutrient agar for confirmation of sterility. The dissection and isolation of gut from surface sterilized fruit flies were carried out as described by Thaochan et al. 2010, with minor modifications. Individual surface-sterilized flies were dissected aseptically in a laminar air flow. Individual dissected whole guts were washed thoroughly with sterile distilled water and transferred to a sterile microfuge tube containing 1 mL of physiologically buffered saline and macerated using a micropestle (Tarsons). A 100 μl aliquot of the macerated suspension was plated on nutrient agar. The inoculated plates were incubated at 37 °C for 24– 48 h. Morphologically distinct predominant bacterial colonies from each of the individual flies were purified on nutrient agar, and stored in slants and 20% glycerol stocks at −80 °C for further use. The isolates were subjected to physiological and biochemical characterization as per standard procedures (Holt et al. 2000).

Molecular identification of gut bacterial isolates

Genomic DNA extraction and PCR amplification of 16S rRNA genes

Genomic DNA was extracted from exponentially grown bacterial colonies using the ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research, USA), according to the manufacturer's instructions. The extracted DNA was quantified by recording the absorbance at 260 nm using a UV/VIS spectrometer (Thermo Scientific, nanodrop 2000C). The universal eubacterial primers 27F5 AGAGTTTGATCMTGGCTCAG 3 and 1487R5 TACCTTGTTACGACTTCACC-3′**,** were used to amplify the 16S rRNA gene following the protocol of Heddi et al. 1998. The amplified fragment was sequenced at Bioserve India Ltd., Hyderabad, India.

Sequence analysis and nucleotide sequence accession numbers

The nucleotide sequences from this study were compared with available sequences from NCBI using BLAST to check their percent identity (Altschul et al. 1997). These nucleotide sequences were aligned with already reported 16S rRNA sequences from GenBank using Clustal W sequence alignment editor, Bioedit 7.0.5.3 (Hall 1999). The 16S rRNA contig sequences of 12 different bacterial isolates have been deposited and GenBank accession numbers were obtained.

Phylogenetic analysis

Sequences with a high percent identity score were imported from the NCBI-GenBank database, and aligned using the MUSCLE multiple alignment program with the default alignment parameters (Edgar 2004). The appropriate substitution model (GTR + G) was chosen based on the Akaike information criterion (AIC) using Partition Finder software (Lanfear et al. 2012). The phylogenetic tree was constructed using MrBayes 3.2.2, Bayesian inference method version 1.1.1. (Ronquist et al. 2012). A stop rule was applied on the run when that value was reached to 0.01, which occurred on the 740,000 Markov Chain Monte Carlo (MCMC) generations with two incrementally heated chains. MCMC started from a random tree and sampling one of every 500 generations, with the first 370 (25%) of the trees discarded as burn-in out of 1480 trees. The remaining trees were subjected to generate a majority-rule consensus tree, the resulting Bayesian inference tree was imported in FigTree version 1.9.3. (Rambaut 2016).

Results

Characterization of gut associated bacteria from fruit flies

The morphological characters of the colony viz., colour, shape and Gram staining were recorded for

Table 1 Physiological characteristics of bacterial isolates obtained from fruit fly guts

Isolates	Growth at pH						Growth at NaCl concentrations				Growth at Temperatures			
	4	5	6	7	8	9	1%	4%	7%	9%	4 °C	27 °C	37 °C	50 °C
BC1	−	++	++	+++	++	+++	+++	++	+	−	−	+++	+++	++
BC2	−	++	+++	+++	+++	+++	+++	++	−	−	−	+++	+++	++
BC3	−	−	++	+++	+++	++	+++	++	++	+	−	+++	+++	++
BC4	−	−	+++	++	++	++	+++	++	−	−	+	+++	+++	−
BC5	−	−	+++	+++	+++	++	+++	+++	−	−	−	+++	+++	+++
BC6	−	++	+++	+++	++	++	+++	++	+	−	−	+++	+++	++
BC7	−	−	+++	++	++	++	+++	+	+	−	+	+++	+++	−
BC8	−	+++	+++	+++	+++	+++	+++	++	+	−	−	+++	++	++
BC9	−	+	++	+++	+++	++	+++	++	+	−	−	+++	+++	+
BC10	−	++	++	++	++	+	+++	++	+	−	+	+++	++	−
BC11	−	++	+++	++	++	+	+++	++	+	−	−	+++	++	+
BC12	++	++	+++	++	++	++	+++	+	+	−	−	+++	++	+

- No growth, + Low growth, ++ Medium growth, +++ High growth

Table 2 Morphological and biochemical characteristics of bacterial isolates obtained from fruit fly guts

Characteristics	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8	BC9	BC10	BC11	BC12
Colour	Dull grey	Creamy white	White	Yellow	White	White	White	White & opaque	White	White & opaque	White	Opaque, mucoid
Shape	Rod	Cylindrical rod	Cocci	Circular rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	−	−	+	−	+	+	+	−	+	−	−	−
Indole Production	+	+	−	−	−	−	−	+	−	−	−	−
Lactose Utilization	−	+	+	+	+	+	+	−	−	+	+	±
Xylose	−	+	−	−	+	−	−	−	−	+	+	+
Maltose	−	+	−	−	+	−	−	−	−	+	+	+
Fructose	+	+	+	−	+	+	+	±	+	+	+	+
Galactose	+	+	+	−	+	+	−	+	−	+	+	+
Raffinose	−	+	−	−	+	±	+	−	−	+	±	+
Trehalose	−	+	−	−	+	−	−	−	−	+	+	+
Sucrose	±	+	+	−	+	+	+	−	−	+	+	+
Arabinose	−	+	−	−	+	±	+	−	−	+	+	+
Salicin	±	+	−	−	+	+	+	−	+	+	+	+
Dulcitol	−	±	−	−	−	−	−	−	−	±	−	+
Inositol	+	+	+	−	−	−	−	−	−	±	−	+
Sorbitol	−	+	±	−	+	−	−	−	−	+	+	+
Mannitol	+	+	+	−	+	+	+	−	−	+	+	+
Rhamnose	±	+	±	−	+	+	+	−	−	+	−	+
Cellobiose	−	+	−	−	+	+	+	−	−	+	+	+
D-Arabinose	−	+	−	−	−	−	−	−	−	+	+	+
Citrate utilization	+	+	+	−	+	+	+	−	−	+	+	±
Esculin hydrolysis	±	+	±	−	+	+	+	−	+	±	+	−
Malanate utilization	−	+	±	−	±	−	−	−	−	+	−	−
ONPG utilization	−	+	±	−	−	+	+	−	−	+	+	+

+ Positive reaction, − Negative reaction, ± Intermediate reaction

all the isolated gut bacteria (Table 2). The physiological (Table 1) and biochemical (Table 2) data were compared as per Bergey's Manual of Determinative Bacteriology (Holt et al. 2000) and further corroborated with their molecular identity (Table 3). The isolates BC1, BC2, BC3 and BC4 obtained from the gut of *B. dorsalis* male flies were identified as *Providencia rettgeri, Klebsiella oxytoca, Enterococcus faecalis,* and *Pseudomonas aeroginosa,* respectively. The isolates BC5, BC6 and BC7 isolated from the gut of *B. cucurbitae* males were identified as *Bacillus cereus, Bacillus licheniformis* and *Bacillus subtilis*. The isolates BC8 and BC9 obtained from the gut of *B. cucurbitae* females were identified as *Morganella morganni* and *Bacillus pumilis* respectively, while the isolates BC10, BC11 and BC12 obtained from the gut of *B. dorsalis* females were identified as *Enterobacter cloacae, Enterobacter asburiae* and *Citrobacter freundii*. Based on the 16S rRNA sequence analysis, the gut bacterial diversity was assigned to eight bacterial genera and twelve species. Among 12 bacterial species, eight species belonged to the Phylum Proteobacteria (66.7%) and comprised mainly of the families Enterobacteriaceae and Pseudomonadaceae. Isolates belonging to Phylum Firmicutes (33.3%) were distributed amongst the families Enterococcaceae and Bacillaceae (Table 1). The isolates *E. asburiae* (KP339861) and *E. cloacae* (KP823456) had a low percent identity (86%), which indicates a possibility of a new species or higher rates of divergence in a particular group.

Phylogenetic analysis

For phylogenetic analyses, three best BLAST hits per sequence were retained. A total of 39 non-redundant sequences were retrieved and aligned with an outgroup sequence of the species *Acidomicrobium ferrooxidans* (Accession number U75647). The 16S rRNA gene dataset alignment was 1592 positions long including the gapped regions. In the final alignment, there were a total of 803 parsimony informative sites. The phylogenetic distribution of the overall gut bacterial diversity using Bayesian Inference Method is shown in the reconstructed tree Fig. 1. The results revealed that the bacterial isolates of fruit flies could be separated into two distinct main monophyletic groups viz., Clade A: Proteobacteria (which comprises of 7 isolates) and Clade B: Firmicutes (which comprises of 5 isolates). Both main groups confirmed with higher Posterior Probability (PP) values equal to 1. In clade A, *Enterocccus faecalis, Bacillus pumilis* and *Bacillus subtilis* matched with other members of the same species (PP =1), whereas *Bacillus licheniformis* and *Bacillus cereus,* grouped with lower support (PP > =53). In clade B, the Proteobacteria were represented by 7 recovered groups, the species viz., *P. aeroginosa, M. morganii, K. oxytoca* and *C. freundii* were detected with higher PP > =0.99 except *P. rettgeri* (PP = 0.58) species. It was observed that *E. asburae* and *E. cloaceae* species were not able to form the distinct clades due to the low percent identity scores.

Table 3 Identity of gut bacterial isolates based on 16S rRNA gene identity

Fruit fly species	Isolate	GenBank Accession no	Identification	Family	Phylum
B. dorsalis ♂	BC1	KT732782	*Providencia rettgeri*	Enterobacteriaceae	Proteobacteria
B. dorsalis ♂	BC2	KT873255	*Klebsiella oxytoca*	Enterobacteriaceae	Proteobacteria
B. dorsalis ♂	BC3	KP339858	*Enterococcus faecalis*	Enterococcaceae	Firmicutes
B. dorsalis ♂	BC4	KP403282	*Pseudomonas aeruginosa*	Pseudomonadaceae	Proteobacteria
B. cucurbitae ♂	BC5	KP403285	*Bacillus cereus*	Bacillaceae	Firmicutes
B. cucurbitae ♂	BC6	KP403286	*Bacillus licheniformis*	Bacillaceae	Firmicutes
B. cucurbitae ♂	BC7	KP676386	*Bacillus subtilis*	Bacillaceae	Firmicutes
B. cucurbitae ♀	BC8	KP403283	*Morganella morganii*	Enterobacteriaceae	Proteobacteria
B. cucurbitae ♀	BC9	KP403284	*Bacillus pumilis*	Bacillaceae	Firmicutes
B. dorsalis ♀	BC10	KP823456	*Enterobacter cloacae*	Enterobacteriaceae	Proteobacteria
B. dorsalis ♀	BC11	KP339861	*Enterobacter asburiae*	Enterobacteriaceae	Proteobacteria
B. dorsalis ♀	BC12	KT732780	*Citrobacter freundii*	Enterobacteriaceae	Proteobacteria

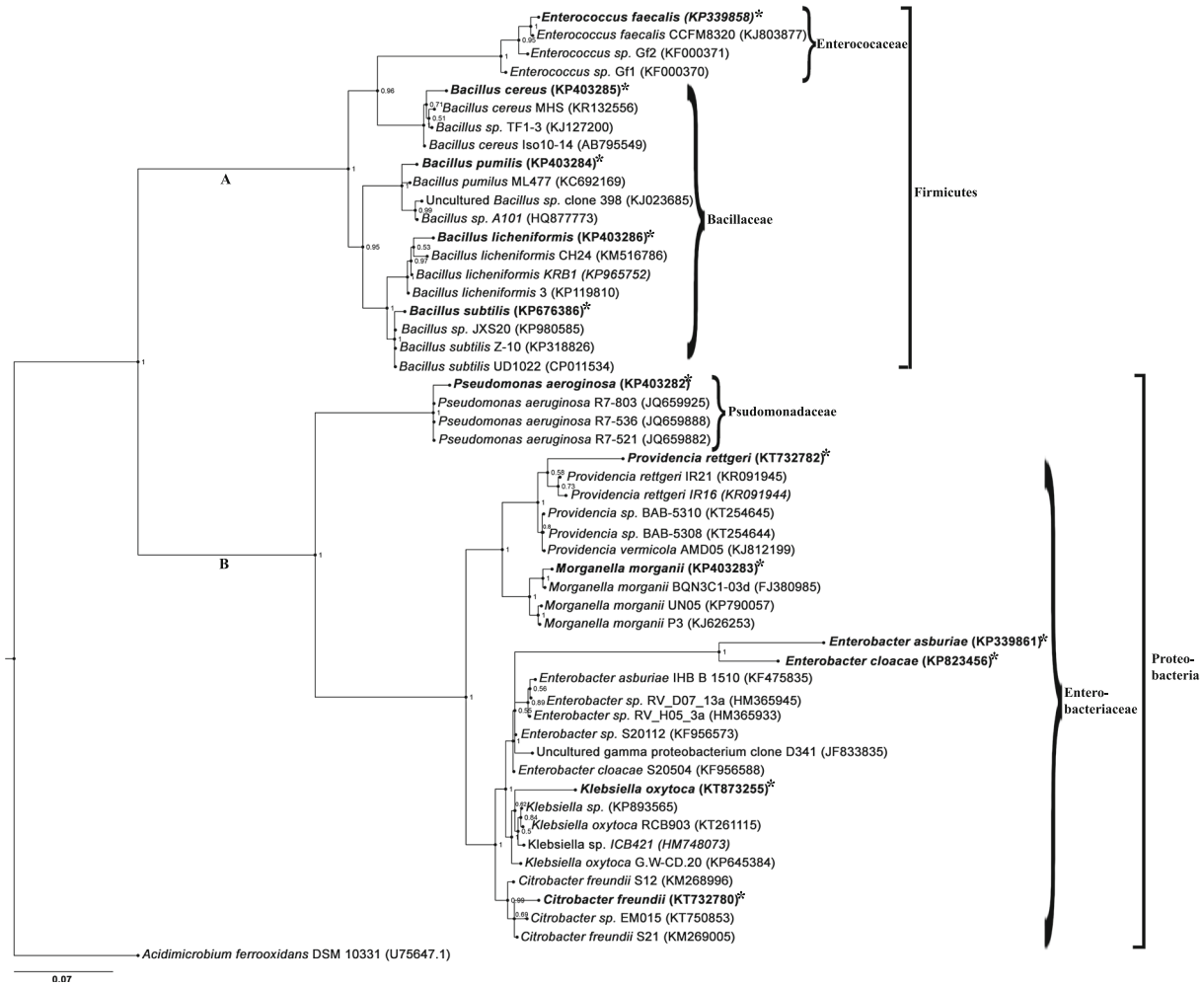

Fig. 1 Phylogenetic Bayesian inference tree based on partial 16S rRNA gene sequences of cultivable bacterial isolates obtained from this study and sequences available in GenBank. Note: Name of isolates from this study and their GenBank accession numbers in brackets are indicated in bold with an * sign. The scale bar indicates the number of substitutions per nucleotide position. Numbers on nodes indicate support for each node ≥0.50 with Posterior Probability values

Discussion

Diverse microorganisms reside in the guts of insects and have developed different interactions with their insect hosts (Engel and Moran 2013). These interactions help in the survival and fitness of insects in the natural environment. Bacteria associated with different species of Tephritidae have been studied earlier (Kuzina et al. 2001; Behar et al. 2008; Thaochan et al. 2010; Prabhakar et al. 2009; Khan et al. 2013). The occurrence of eighteen different bacterial species belonging to the family Enterobacteriaceae, Bacillaceae and Pseudomonadaceae were reported from Mexican fruit flies *Anastrepha ludens* (Diptera: Tephritidae) (Kuzina et al. 2001), The most common bacterial species associated with *Bactrocera* flies were *C. freundii*, *E. cloacae* and *K. oxytoca* (Behar et al. 2008; Drew and Lloyd 1991). But the gender wise bacterial diversity is not evident. The present study was therefore carried out to explore the cultivable bacterial diversity associated with the guts of male and female fruit fly species viz., *Bactrocera dorsalis* and *Bactrocera cucurbitae.* Isolation, characterization and polyphasic identification of the bacterial isolates revealed the predominance of bacteria belonging to families Enterobacteriaceae, Bacillaceae and lesser occurence of bacteria belonging to families Pseudomonadaceae and Enterococcaceae. Various genera of fruit flies are known to harbour stable

bacterial communities belonging to family Enterobacteriaceae in their digestive system *viz., Bactrocera* (Thaochan et al. 2010; Wang et al. 2011), *Anastrepha* (Kuzina et al. 2001) and *Ceratitis* (Behar et al. 2005; Behar et al. 2008). Members of Enterobacteriaceae play an important role in fruit fly fitness (Ben-Ami et al. 2010). In present study the guts of *B. dorsalis* males and females were predominantly colonized by members of family Enterobacteriaceae. These bacteria have been found to fix atmospheric nitrogen and detoxifiy defensive coniferous compounds like monoterpenes, diterpene acids and phenolic resins in the guts of bark beetle *Dendroctonus frontalis* (Vasanthakumar et al. 2006; Morales-Jimenez et al. 2012). The occurrence of *Klebsiella oxytoca* in the Mediterranean fruit fly *Ceratitis capitata* has been reported by (Yuval et al. 2010). The addition of this bacterium to the post irradiation diet was found to significantly improve the performance of sterile males.

A significant observation in this study is that the guts of *B. cucurbitae* are colonized predominantly by members of the family Bacillaceae. Previously the abundance of *Bacillus* species has been reported from the guts of *Apis mellifera ligustica, Apriona germari* and *Lymantria dispar* (Yuan et al. 2009; Zhang et al. 2004; Broderick et al. 2004).

Most of the isolated bacterial species are common inhabitants of soil, water and decomposing organic matter and therefore their presence in the guts of natural populations are justified. But the presence of these species in laboratory reared larvae is quite intriguing. It is to be examined if the species are vertically transmitted through the maternal route. The attractiveness of adult fruit flies to bacterial species has been attributed to the presence of chemical constituents (Robacker and Bartelt 1997; Drew and Fay 1988; Lee et al. 1995). Chemical volatiles extracted from the gut bacteria of Mexican fruit flies were assayed to establish their attractiveness to the host. These chemical volatiles were subsequently used in fruit fly lures for effective management (Robacker et al. 1996, 2000). The presence of *Klebsiella oxytoca* has also been recorded to increase the competition of male fruit flies in case of *Anastrepha ludens* (Yuval et al. 2010). Since this study has clearly established the gender specific presence of gut bacterial species in two economically important fruit fly species, the role of gender specific bacterial volatiles would be an interesting researchable issue for the future.

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