Research Article

Received: 10 February 2015 Revised: 3 July 2015 Accepted article published: 24 August 2015 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4102

Diversity of bacterial communities in the midgut of *Bactrocera cucurbitae* (Diptera: Tephritidae) populations and their potential use as attractants

Ashok B Hadapad,[a] Chandra S Prabhakar,[a,b] Snehal C Chandekar,[a] Jyoti Tripathi[c] and Ramesh S Hire[a]*

Abstract

BACKGROUND: The microbiota plays an important role in insect development and fitness. Understanding the gut microbiota composition is essential for the development of pest management strategies. Midgut bacteria were isolated from nine wild *B. cucurbitae* populations collected from different agroecological zones of India. These isolates were further studied for attractant potential of fruit fly adults, and the chemical constituents in the supernatants of gut bacteria were analysed.

RESULTS: Twenty-six bacterial isolates belonging to the families Enterobacteriaceae, Bacillaceae, Micrococcaceae and Staphylococcaceae were isolated and identified on the basis of *16S rRNA* gene sequence analysis. The dominant species in the midgut of melon fly were from the genera *Enterobacter* (34.6%), *Klebsiella* (19.2%), *Citrobacter* (7.7%), *Bacillus* (15.4%) and *Providencia* (7.7%), and 3.8% each of *Micrococcus*, *Staphylococcus*, *Leclercia* and *Exiguobacterium*. *Bactrocera cucurbitae* and *B. dorsalis* adults were significantly attracted to bacterial whole cell cultures and their supernatants in the fruit fly attraction bioassays. *Bacillus cereus*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Providencia* species attracted both male and females of *Bactrocera* species. The supernatants of *Klebsiella*, *Citrobacter* and *Providencia* species attracted a significantly greater number of females than males. The most abundant chemical constituents in supernatants of *K. oxytoca* and *C. freundii* were 3-methyl-1-butanol, 2-phenylethanol, butyl isocyanatoacetate, 2-methyl-1-propanol and 3-hydroxy-2-butanone, as identified by gas chromatography–mass spectrometry.

CONCLUSIONS: The bacterial endosymbionts associated with melon fly exhibited attractant potential which could facilitate eco-friendly insect control strategies.

Keywords: *Bactrocera cucurbitae*; *16S rRNA* gene; gut microbiota; fruit fly attractant; volatile compounds

1 INTRODUCTION

Fruit flies belonging to the genus *Bactrocera* (Diptera: Tephritidae) are major invasive pests and are ranked as the world's most serious pest of horticultural crops. The melon fly, *Bactrocera cucurbitae* (Coquillett), is a major threat to commercial cucurbits and widely distributed in different parts of the world.[1] Generally, plants belonging to the family Cucurbitaceae are preferred hosts of this fly, and it damages over 125 plant species.[1,2] Owing to its vast adaptability, high reproduction potential and invasion capacity, the melon fly has been the subject of a worldwide pest management programme.[3] Several management strategies are being employed for the control of fruit flies, while the success and effectiveness of different strategies are greatly influenced by various natural factors. The sterile insect technique (SIT) is an environmentally friendly and species-specific method of pest control based on the release of large numbers of sterile insects.[4] This method has been successfully implemented against various insect pest species, including the melon fly.[3,5]

Arthropods are known to harbour various endosymbionts that have close interactions either intracellularly or extracellularly.[6] The bacterial endosymbionts play an important role in host nutrition, development, fitness, survival, modulation of immune responses and communication.[7–11] A vast range of gut bacteria have been isolated and identified from different insect orders.[10] The presence of such an enormous bacterial diversity within insect

* *Correspondence to: Ramesh S Hire, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India E-mail: rshire@barc.gov.in; ramesh.hire@gmail.com*

a *Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai, India*

b *Department of Entomology, Bihar Agricultural University, Sabour, Bhagalpur, Bihar, India*

c *Food Technology Division, Bhabha Atomic Research Centre, Trombay, Mumbai, India*

digestive tracts is due to their different feeding habits, physicochemical properties and gut structures.[6,10,12] In Tephritidae, different bacterial species belonging to the families Enterobacteriaceae, Pseudomonadaceae, Vibrionaceae, Micrococcaceae, Staphylococcaceae and Bacillaceae were predominantly isolated from the gut of *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus* and *Rhagoletis* species.[13–21] Many studies on fruit flies have provided valuable information on the role of microorganisms in host plant interaction and fruit fly biology.[10,14,15,22]

Certain volatile components of fruit fly's gut bacteria play a vital role in fruit fly behaviour as either feeding or ovipositional stimulants[15,22–24] and are being exploited in pest management in the form of traps or baits.[25,26] Enteric bacteria such as *Enterobacter cloacae*, *E. agglomerans*, *Klebsiella oxytoca* and *Citrobacter freundii* isolated from the alimentary tract of wild and laboratory-reared *Dacus dorsalis* (Hendel) showed an olfactory attraction response by *D. dorsalis* adults.[14] Culture filtrates of *K. pneumoniae* and *C. freundii* isolated from the gut of the Mexican fruit fly *Anastrepha ludens* (Loew) attracted this species.[25,27] Similarly, *Bacillus cereus*, *Enterococcus faecalis*, *E. cloacae* and *C. freundii* isolated from gut of *B. dorsalis* significantly attracted *B. dorsalis* adults.[21] The attraction of fruit fly adults to gut bacterial odours is due to the volatile chemical compounds emitted during the fermentation.[22] Several volatile compounds, including alcohols, ammonia, pyrazines, sulphur compounds, ketones, acids, phenols and amines, have been identified from various gut bacteria and found to attract fruit fly adults.[27–31] Volatile compounds such as 3-methyl-1-butanol, 2-phenylethanol, 2-methyl-1-propanol, phenol and 2,5-dimethylpyrazine were more frequently identified from gut bacteria and were shown to attract insects.[22,29,32,33] Thus, insect-associated bacterial symbionts and their byproducts could be used for the development of novel biocontrol agents and may act as semiochemicals for the management of insect pests and vectors. In the present investigation, the composition and diversity of the microbial community in the midgut of melon fly obtained from nine wild populations were identified using *16S rRNA* gene sequence analysis and further assessed for their attractant potential. The chemical constituents in supernatants of melon fly gut bacteria were studied through gas chromatography–mass spectrometry (GC-MS).

2 MATERIALS AND METHODS

2.1 Fruit fly collection and storage

Male adult flies of *B. cucurbitae* were collected from nine locations distributed in different states of India using cue-lure traps (Nauroji Stonehouse fruit fly trap, Navasari Agriculture University, Gujarat, India) during the period 2012–2013 (Table 1). For each location, 4–5 spots in vegetable fields were identified, traps were installed and melon fly specimens were collected after 24 h. The collected melon flies were placed individually in plastic vials to prevent cross-contamination of bacteria between flies of the same location as well as from different locations. Collected flies were stored at −20 °C in vials containing 800 µL of absolute ethanol to protect them from regurgitation within the tubes.

2.2 Dissection and isolation of bacteria from the gut of melon fly

Five adult male flies of *B. cucurbitae* were randomly selected from each location to isolate the gut bacterial flora. Before dissection, flies were surface sterilised by submergence in a sequence of 70% ethanol for 1 min and 0.5% sodium hypochlorite for 1 min and then washed twice (1 min each) in sterile distilled water.[19] The individual surface-sterilised flies were dissected aseptically under laminar air flow. The insect midgut portion extending in-between crop to the joints of malpighian tubules were separated and transferred to a sterile microcentrifuge tube containing 1 mL of saline. After the appropriate serial dilutions, 100 µL of midgut content was plated on tryptone soy agar (TSA) and peptone yeast extract agar (PYEA) media using a sterile glass spreader and incubated at 37 °C for 24 h. A qualitative assessment of different types of colony growing on each plate of TSA and PYEA was made after 24 h of incubation. Predominant bacterial colonies showing a distinct appearance were purified by subculturing twice using the quadrant streaking method. The purified colonies were subjected to morphological, biochemical and molecular characterisation.[34] The glycerol stocks of purified bacterial colonies were preserved in −70 °C for further use.

2.3 Molecular characterisation of gut microbiota

2.3.1 Total DNA extraction

The individual, purified bacterial colony was grown in 10 mL of peptone yeast extract broth (PYEB) at 28 °C on a rotary shaker at 120 rpm (Orbitek; Scigenics Biotech, Chennai, India). The culture was harvested after 24 h of incubation, and the pellets were prepared by centrifugation at 10 000 × *g* for 5 min. The genomic DNA was isolated as per the protocol described.[35] The DNA pellet was dissolved in 10 µL of TE buffer pH 8.0 (10 mM of Tris HCl pH 7.4, 1 mM of EDTA pH 8.0) containing RNase (10 µg mL^{-1}). The quantity and quality of DNA was determined by NanoDrop 2000 (Thermo Scientific, Wilmington, DE) spectophotometric analysis at 260 nm and agarose gel (0.8%) electrophoresis.

2.3.2 PCR amplification of the gut bacterial 16S rRNA gene

PCR amplification was carried out using universal bacterial primer pairs Eub27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Eub1492R (5′-ACG GCT ACC TTG TTA CGA CTT-3′).[36] Amplification was performed in 20 µL reactions in a thermal cycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany). Each reaction tube contained 2 µL of 10× reaction buffer with $MgCl_2$ (15 mM), 0.5 µL of dNTPs (25 mM), 0.5 µL of each primer (25 mM), 0.5 µL (5 U μL^{-1}) of *Taq* DNA polymerase and 1 µL of template DNA. PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final extension period of 7 min at 72 °C. PCR products were resolved on an agarose gel (1.4% agarose, 1 × TBE) stained with ethidium bromide. Negative controls (no DNA added) were routinely included as a contamination check, and no products were obtained from these controls. The PCR amplicon was purified with a Nucleospin purification column (Macherey-Nagel GmBH & Co., Düren, Germany). The purified PCR products were sequenced in both directions using an ABI 3730xl cycle sequencer. All bacterial sequences were submitted to GenBank (Table 1).

2.3.3 Phylogenetic analysis

Sequences were compared with the GenBank database using the Basic Local Alignments Search Tool (BLAST) to identify their closely related *16S rRNA* gene sequences.[37] The nucleotide sequences from this study were aligned with reported *16S rRNA* gene sequences using ClustalW2. The isolates were identified on the basis of 99% similarity between bacterial sequences of this study with NCBI database sequences. The phylogenetic tree was

Table 1. Collection and identification of bacterial colonies isolated from melon fly *Bactrocera cucurbitae* obtained from different geographical locations of India

State	Location	Lab accession	Year of collection	Latitude, longitude	Bacterial species and strain number	GenBank accessions
Maharashtra	Dahanu	BCD	2012	19° 76′ N, 72° 97′ E	*Enterobacter hormaechei* BCD1	KC154049
					Providencia rettgeri BCD2	KC154050
					Enterobacter hormaechei BCD3[b]	KC154051
					Providencia rettgeri BCD4[b]	KC154052
					Klebsiella pneumoniae BCD5	KC154053
	Gamma field[a]	BCG	2012	19° 01′ N, 72° 93′ E	*Enterobacter cancerogenus* BCG1	KF224913
					Enterobacter hormaechei BCG4	KF224914
					Enterobacter hormaechei BCG5	KF224915
Himachal Pradesh	Solan Nauni Farm	BCSN	2012	30° 90′ N, 77° 09′ E	*Bacillus cereus* BCSN2	KF224921
					Bacillus cereus BCSN3	KF224922
	Solan Pandah Farm	BCSP	2012	30° 85′ N, 77° 17′ E	*Bacillus megaterium* BCSP2	KF224923
					Enterobacter sp. BCSP3	KF224924
	Palampur	BCP	2012	32° 11′ N, 76° 53′ E	*Micrococcus luteus* BCP1	KF224920
Jammu and Kashmir	Srinagar	BCS	2012	34° 08′ N, 74° 79′ E	*Staphylococcus alettae* BCS1	KF224916
					Exiguobacterium aurantiacum BCS2	KF224917
Tamil Nadu	Coimbatore	BCC	2013	11° 01′ N, 76° 95′ E	*Enterobacter hormaechei* BCC2	KF224909
					Enterobacter hormaechei BCC3	KF224910
					Citrobacter freundii BCC4	KF224911
					Citrobacter freundii BCC5	KF224912
Kerala	Kasaragod	BCK	2013	12° 50′ N, 75° 00′ E	*Leclercia adecarboxylata* BCK1	KF224918
					Bacillus cereus BCK3	KF224919
Karnataka	Bengaluru	BCB	2012	12° 98′ N, 77° 58′ E	*Klebsiella oxytoca* BCB1	KF224904
					Klebsiella variicola BCB2	KF224905
					Enterobacter hormaechei BCB3	KF224906
					Klebsiella oxytoca BCB4	KF224907
					Klebsiella variicola BCB5	KF224908

[a] Mumbai.
[b] Female origin; the bacterial species were identified on the basis of *16S rRNA* sequences.

constructed using neighbour-joining (NJ) algorithms in MEGA v.5, with 1000 bootstrap replicates to compute the standard error of distance estimates.[38] The Jukes–Cantor model was used to calculate nucleotide substitution distances.[39]

2.4 Bacteria cultivation and fruit fly attractant assays

Twenty-six gut bacterial cultures were grown in a 100 mL flask containing 25 mL of PYEB media. A single colony of each bacterial culture was inoculated and incubated on a rotary shaker (28 °C, 150 rpm) for 72 h. A 5 mL aliquot was removed from each grown culture and designated as whole cell culture, and the remaining 20 mL was filtered with filter paper (Whatman No. 40) and designated as supernatant. The whole cell culture and supernatant from all isolates were used to determine the attractive potential. The uninoculated PYE broth was used as control.

All attractant assays were conducted in the laboratory as per the method described,[25] with slight modification. In brief, whole cell cultures, supernatants and uninoculated PYE broth (200 µL each) were loaded onto a filter paper disc (~3 cm diameter) in a petri dish and placed in separate insect cages (45 × 45 × 45 cm). These petri dishes containing treatment and control were kept diagonally from each other at two corners of the cage. The *B. cucurbitae* and *B. dorsalis* adults were maintained at 26 ± 2 °C and 75% RH. Mass-produced adults (13 generations) of both species were used for this experiment. The bacterial cultures and control media were placed in insect cages (45 × 45 × 45 cm) containing ~200 fruit flies (sex ratio about 1:1; 10–12 days old) of *B. cucurbitae* and *B. dorsalis* in separate cages for each experimental set-up. The number of fly visits per disc was scored for 10 min for each isolate. The experiments were conducted in four replicates with different day's culture and different sets of insects. The treatment/control (T/C) ratio was calculated on the basis of the total numbers of adults counted on treatment filter paper to the counts in PYE broth as reported earlier.[25] Differences among the number of attracted adults in treatments and control were subjected to analysis of variance (ANOVA, $P < 0.05$) and compared using Tukey's HSD multiple comparison test (SPSS 12.0 for Windows).

2.5 Identification of volatile compounds through GC-MS

The supernatants of gut bacterial isolates *Klebsiella oxytoca* BCB1 and *Citrobacter freundii* BCC5 were used for identification of volatile compounds through solid-phase microextraction in the headspace, which was coupled with gas chromatography and mass spectrometry detection (HS-SPME-GC-MS). The cultures were grown in a 500 mL flask containing 100 mL of PYEB media for 72 h as described above. The bacterial cultures were harvested by centrifugation at 10 000 × *g* for 15 min at 4 °C. The supernatant was filtered through a 0.45 µm membrane filter by using a vacuum pump (Millipore Corporation, Bedford, MA) and further used for GC-MS analysis.

Volatile compound profiles of the bacterial supernatants and culture medium (PYEB) were studied using HS-SPME-GC-MS as per

the method described earlier, with slight modifications.[40] In brief, 15 mL of each bacterial supernatant and control medium (PYEB) was taken in separate SPME vials (40 mL) containing 4.5 g of NaCl. These samples were equilibrated for 30 min at 30 °C with continuous stirring, and headspace was subsequently extracted by preconditioned PDMS/DVB/CAR fibres (Supelco, West Chester, PA) for 20 min at 30 °C. After extraction, fibres were desorbed (270 °C) in the injection port of a Shimadzu GC-MS instrument (Shimadzu Corp., Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5 (J&W Scientific, Folsom, CA) capillary column [(5% phenyl)-methylpolysiloxane, length 30 m, i.d. 0.25 mm, film thickness 0.25 µm]. The operating conditions were: column temperature programmed from 40 to 200 °C at a rate of 4 °C min, held at the initial temperature and at 200 °C for 5 min, and further to 280 °C at a rate of 10 °C/min^{-1}, held at the final temperature for 20 min; injector and interface temperatures 210 and 280 °C respectively; carrier gas helium (flow rate 0.9 mL min^{-1}), ionisation voltage 70 eV, electron multiplier voltage 1 kV. Peaks were tentatively identified by comparing their mass fragmentation pattern with standard spectra available in the spectral library (Wiley/NIST Libraries) of the instrument, as well as by comparing the Kovats index (KI) values of the compounds with literature data.

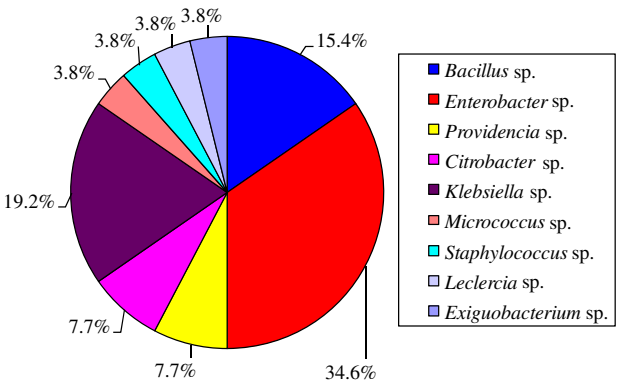

Figure 1. Percentage incidence of bacterial colonies in the midgut of melon fly, *Bactrocera cucurbitae*, collected from nine geographical locations (five adult flies per location).

3 RESULTS

3.1 Isolation and identification of gut bacteria

Melon fly (*B. cucurbitae*) samples collected from nine locations of different states of India are presented in Table 1. Twenty-six morphologically distinct bacterial cultures have been isolated from the midgut of the wild melon fly population using two bacteriological media (PYEA and TSA). The physical characteristics of the colonies were similar when grown on both culture media with circular, convex, flat, white mucoid and yellow colonies. No fungi or yeasts were recovered. Gram-negative, non-motile, rod- and coccus-shaped bacteria were the major bacteria in the gut of melon fly. Out of the 26 isolates, six were gram-positive, including four bacilli and two cocci, while 20 were gram-negative, comprising enterobacteria.

3.2 *16S rRNA* gene sequence analysis

Based on the *16S rRNA* gene sequence analysis, nine genera and 14 species were identified according to percentage similarity of the NCBI database (Table 1). Among the 26 bacterial isolates, nine *Enterobacter* sp. (34.6%), five *Klebsiella* sp. (19.2%), four *Bacillus* sp. (15.4%), two *Citrobacter* sp. (7.7%), two *Providencia* sp. (7.7%) and one (3.8%) each of *Micrococcus*, *Staphylococcus*, *Leclercia* and *Exiguobacterium* sp. were identified (Fig. 1). Most of the isolates belonged to the family Enterobacteriaceae, which comprised 73.1% of all the isolates.

Phylogenetic analysis of *16S rRNA* gene sequences of 26 gut bacteria and 28 bacterial *16S rRNA* gene sequences obtained from GenBank revealed that sequences of present study clustered with genera and species of reported sequences. Overall sequences were grouped in four clusters, in conformity with the bacterial families Enterobacteriaceae, Micrococcaceae, Staphylococcaceae and Bacillaceae (Fig. 2). Out of 26 gut bacteria, the majority (19) clustered with members of Enterobacteriaceae. One bacterial isolate each clustered with Micrococcaceae and Staphylococcaceae. Four bacterial sequences showed aggregation with a member of the Bacillaceae family. The genetic distance within the members of the families Enterbacteriaceae, Micrococcaceae, Staphylococcaceae and Bacillaceae was 0.020, 0.006, 0.003 and 0.031 nucleotides per site respectively (Fig. 2). The pairwise genetic distance between families ranged from 0.067 between Staphylococcaceae and Bacillaceae to 0.229 between Enterbacteriaceae and Bacillaceae.

3.3 Attractant study

The bacterial isolates of melon fly gut attracted a significant number of adults of both species of *Bactrocera* by comparison with the control. An increase of ~2–7-fold in attraction of adults was observed towards whole cell culture and supernatants of gut bacteria. *B. cereus* BCK3, *E. hormaechei* BCD1, *Klebsiella* sp. (BCB1, BCB4 and BCD5), *Citrobacter* sp. (BCC4 and BCC5) and *Providencia* sp. (BCD2 and BCD4) were more effective in attracting both male and female *B. cucurbitae* and *B. dorsalis* adults (Figs 3A and B). Whole cell cultures of *K. pneumoniae* (BCD5), *K. oxytoca* (BCB1 and BCB4) and *C. freundii* (BCC4 and BCC5) attracted significantly greater numbers of male and female *B. cucurbitae* ($P < 0.05$) and *B. dorsalis* ($P < 0.05$) (Fig 3A). Similarly, whole cell cultures of *Bacillus* and *Providencia* species also attracted both sexes of *Bactrocera* species. The supernatants of bacterial isolates were also attractive to both male and female *B. cucurbitae* and *B. dorsalis* adults, whereas some of the supernatants, such as *C. freundii* (BCC4 and BCC5), *K. oxytoca* (BCB1 and BCB4) and *P. rettgeri* (BCD2), attracted a greater number of females than males of both *Bactrocera* species ($P < 0.05$) (Fig 3B). Overall, the melon fly gut bacteria attracted both male and females of *Bactrocera* species under laboratory conditions.

3.4 Volatile compounds in supernatants of gut bacteria

The chemical constituents in supernatants of *Klebsiella oxytoca* BCB1 and *Citrobacter freundii* BCC5 after 72 h fermentation were identified through GC-MS analysis. The identified volatile compounds mainly consisted of alcohols (four), ketones (four), acids (two), pyrazines (two), phenol, nitrogen-containing (two) and sulphur-containing (one) compounds in these bacterial supernatants (Table 2). Of the 16 identified volatile compounds, the most abundant compounds were 3-methyl-1-butanol, 2-phenylethanol, butyl isocyanatoacetate, 2-methyl-1-propanol and 3-hydroxy-2-butanone (Table 2). Among them, 3-methyl-1-butanol contributes 41.58–49.42% of volatiles in PYE broth containing *K. oxytoca* BCB1 and *C. freundii* BCC5. Some other volatile compounds, such as 2-methyl-3-heptanone, phenol, 3-(methylthio)-1-propanol, phenol, indole, acids, and pyrazines

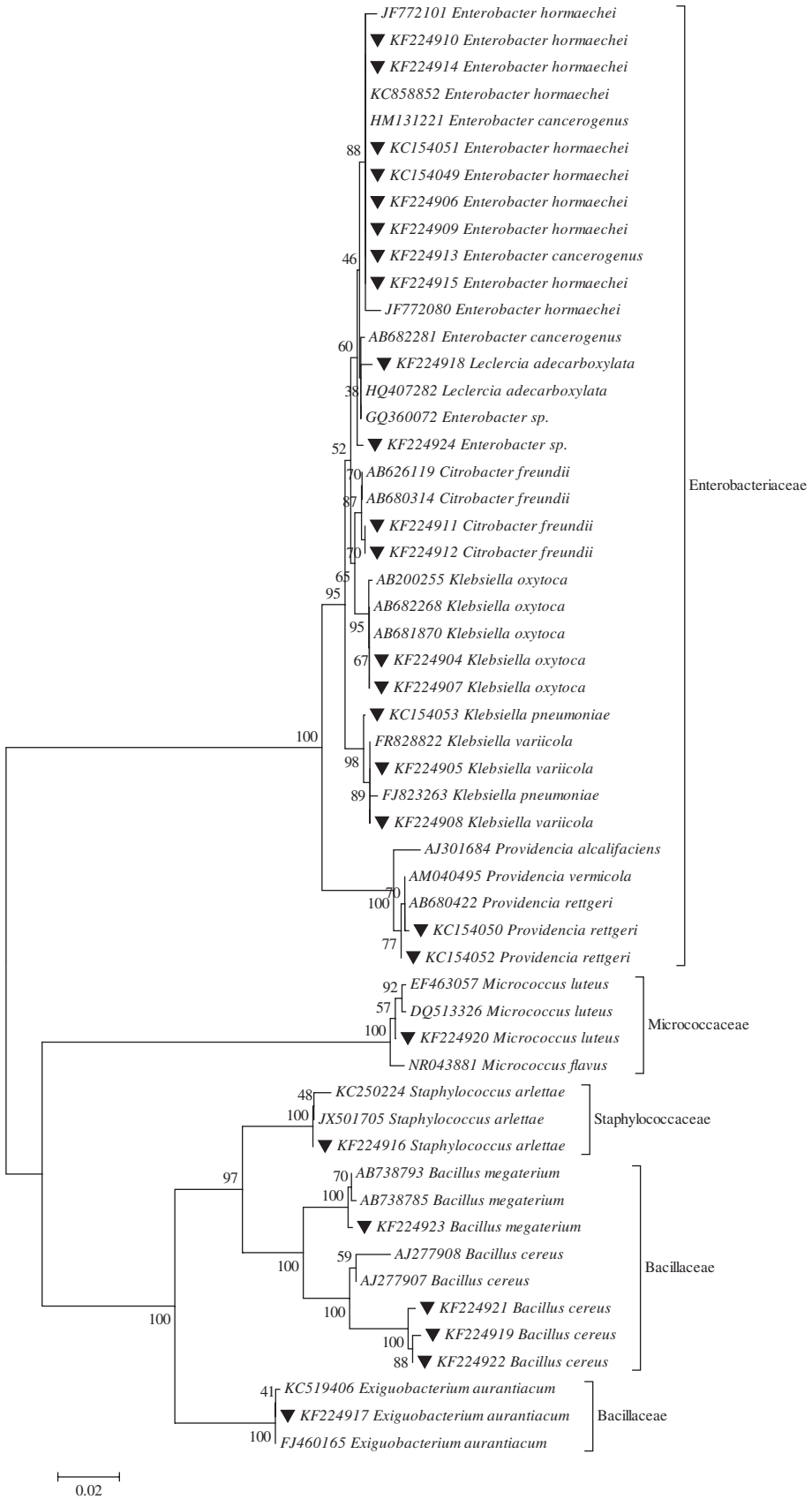

Figure 2. Phylogenetic tree of gut bacteria of *Bactrocera cucurbitae*, based on *16S rRNA* gene sequences. The tree was constructed using the neighbour-joining method, and confidence limits were tested by the bootstrap method (1000 replications). Bacterial isolates sequenced in the present study are marked with the symbol ▼.

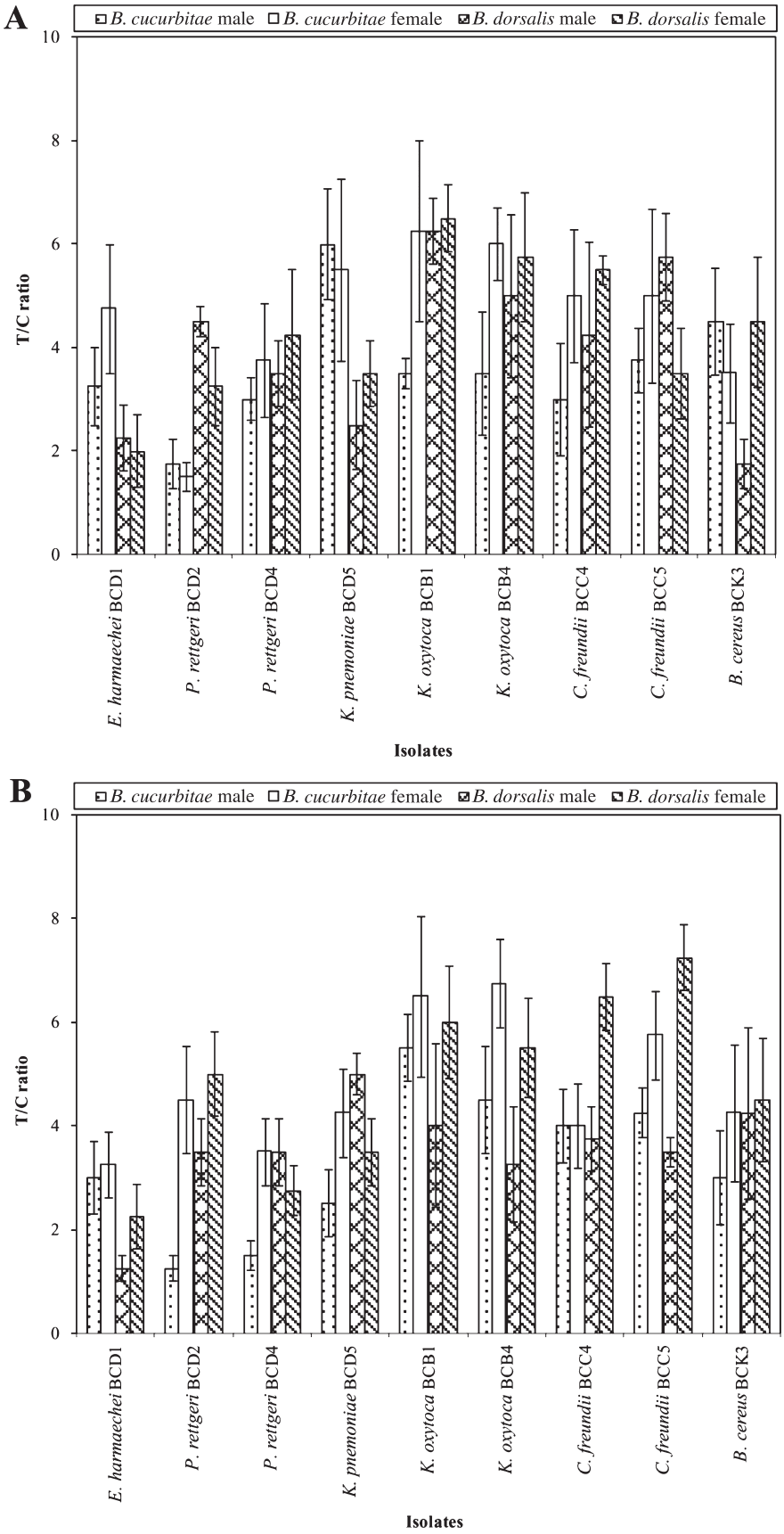

Figure 3. Attraction of *Bactrocera cucurbitae* and *B. dorsalis* adults to whole cell cultures (A) and supernatants (B) of bacterial isolates. The bars indicate the standard error of four replicates compared using Tukey's HSD multiple comparison test.

Table 2. Major volatile compounds identified by GC-MS in supernatants of *Bactrocera cucurbitae* gut bacteria

Compounds	Kovats index[a]	Relative area (%)[b] *Klebsiella oxytoca* BCB1	Relative area (%)[b] *Citrobacter freundii* BCC5
Acetic acid	605	1.19	0.40
2-Methyl-1-propanol	645	1.68	1.66
3-Methyl-1-butanol	740	49.42	41.58
2-methyl-1-butanol	756	0.17	1.95
Butyl isocyanatoacetate	765	6.15	5.96
3-Hydroxy-2-butanone	812	1.94	0.87
2-Methyl-4-heptanone	885	0.13	0.05
2-Methyl-3-heptanone	888	0.22	0.22
3-(Methylthio)-1-propanol	980	0.34	tr
Phenol	985	ND	18.06
2-Hydroxy-4-methyl pentanoic acid	1070	0.19	0.23
2,5-Dimethyl-3-ethylpyrazine	1080	0.02	0.01
2-Nonanone	1090	0.02	0.01
2-Phenylethanol	1120	11.15	7.51
2,3,5-Trimethyl-6-ethylpyrazine	1160	0.12	tr
Indole	1290	ND	13.25

[b]Kovats indices calculated from retention time data obtained on a DB-5 capillary column. ND: not detected; tr: trace amount.

(Table 2), were also identified in supernatants of these bacteria, whereas these volatile compounds were not detected in uninoculated PYEB media. The attraction of *Bactrocera* adults towards supernatants of *Klebseilla* and *Citrobacter* sp. could be due to either individual or a combination of these volatile compounds present in the supernatants.

4 DISCUSSION

Diverse microorganisms inhabit the intestinal tract of insects and have developed different interactions with host insects.[10] These interactions help in the survival and fitness of insects in the environment. In the present study, we isolated and identified the gut microbiota of the melon fly using TSA and PYEA media. Various gut bacteria have been isolated from *Bactrocera cacuminata* (Hering), *B. tryoni* (Froggatt) and other *Bactrocera* species.[18–21] Based on the *16S rRNA* gene sequence analysis, nine genera and 14 species were identified, belonging to the familes Enterobacteriaceae (73.1%), Bacillaceae (15.4%), Micrococcaceae (3.8%) and Staphylococcaceae (3.8%). This indicates that members of the family Enterobacteriaceae mainly harbour in the midgut of the wild melon fly population. In fruit flies, the family Enterobacteriaceae has been reported frequently and is dominant in the guts of Bactrocera,[19–21] *Anastrepha*[16] and *Ceratitis*.[17,41] Members of Enterobacteriaceae play an important role in fruit fly fitness and adult food source.[8] They also possibly act as a stimulus to attract foraging flies to the host fruit tree.[15,28] Overall, wild *B. cucurbitae* populations mainly host *Klebsiella*, *Citrobacter*, *Enterobacter*, *Providencia* and *Bacillus* species.

The bacterial endosymbionts are known to attract the fruit fly population in the laboratory as well as under field conditions[21,26,30,42] In the present study, members of Enterobacteriaceae (*E. hormaechei*, *Klebsiella*, *Citrobacter* and *Providencia* sp.) and Bacillaceae (*B. cereus*) attracted both males and females of *Bactrocera* species. Bacterial species of the family Enterobacteriaceae isolated from wild and laboratory-reared *D. dorsalis* attracted both male and female adults.[14] The bacterial filtrate obtained from *Pseudomonas putida* isolated from the olive fly *B. oleae* (Rossi) also showed attraction to both males and females.[31] Interestingly, the supernatants of *C. freundii*, *K. oxytoca* and *P. rettgeri* cultures attracted significantly more females than males. It has also been observed that *B. dorsalis* adults were attracted to metabolites of Enterobacteriaceae, Enterococcaceae and Bacillaceae. Further, more female flies were captured in the traps baited with the metabolites of these bacteria in the field.[21] The response of male and female fruit fly adults to different conditions of bacterial cultures showed that a higher number of females were attracted to *C. freundii*.[14] This indicates that these bacteria produce volatile compounds that specifically attract female fruit flies.

The attractiveness of fruit fly adults to gut bacteria, mainly due to the presence of several chemical constituents such as ammonia, alcohols, sulphur compounds, ketones, several amines, phenols, pyrazines and acetic acid, has been reported earlier.[27–32] In the present study, 3-methyl-1-butanol, 2-phenylethanol, butyl isocyanatoacetate, 2-methyl-1-propanol and 3-hydroxy-2-butanone were identified from supernatants of *K. oxytoca* BCB1 and *C. freundii* BCC5. These compounds were not detected in uninoculated PYE broth. Similarly, 3-methyl-1-butanol, 2-phenylethanol, several amines, 2-methyl-1-propanol, phenol and 2,5-dimethylpyrazine were previously identified from *Klebsiella* and *Citrobacter* sp.[27,29,32] Some other volatile compounds, including acids, pyrazines, ketones and sulphur- and nitrogen-containing compounds, were also detected in *Klebsiella* and *Citrobacter* sp. supernatants. Moreover, supernatants of *Klebsiella* and *Citrobacter* sp. attracted more female than male *Bactrocera* adults. It has been observed that a greater number of *B. dorsalis* females were attracted to gut bacteria.[14,21] The volatile chemicals such as 3-methyl-1-butanol and ammonia produced by *E. agglomerans* of *A. suspensa* (Loew) and fruits infested with larvae are known to attract female Caribbean fruit fly in laboratory assays.[33] This indicates that volatile compounds identified in the present study might have contributed to attracting either both sexes or only female *Bactrocera* adults. Thus, the compounds of bacterial origin could act as an efficient communication in disrupting insect pest species[22,43] The volatile compounds produced by the gut bacteria vary according to species, bacterial strain, growth media and conditions.[23] The mass production of insect gut bacteria using low-cost media and their volatiles could be used as semiochemicals and/or parapheromones to trap insects under field conditions, and can also be used to enhance the performance of parasitoids and predators.[22,44]

It has also been observed that during the irradiation process the structure of the bacterial population and midgut tissue was affected in the sterile Mediterranean fruit fly, which eventually reduces competitiveness with wild males.[8,45] For example, the family Enterobacteriaceae is the dominant bacterial group in the gut of fruit flies, and among these, *Klebsiella* sp. is the most dominant species in wild flies,[41] which decreased significantly after sterilisation[8]. Further, it has been demonstrated that the competitiveness and fitness of sterile males could be enhanced by supplementing gut bacterial species in the form of probiotic diet.[9,11,46,47] Results of the present study showed that melon-fly-associated bacterial endosymbionts exhibit attractant potential towards *Bactrocera* adults. The volatile compounds identified from the supernatants of gut bacteria could result in the development of novel

bait molecules with immense potential for controlling the fruit fly population.

ACKNOWLEDGEMENTS

We thank Dr SP Kale, Head, NA&BTD, BARC, Mumbai, for support and encouragement. This research was conducted under the Coordinated Research Project (research contract number 17090) supported by the International Atomic Energy Agency (IAEA), Vienna. We thank Dr Mahesh Subramanian, BARC, for critically reading the manuscript. We are grateful to Mr SM Tota and Mr Uttam Sahane, KVK, Kosbad, for help in collection of fruit fly samples.

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