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ORIGINAL ARTICLE

# Attractancy potential of bacterial volatiles from symbiotic bacteria of wild population of *Zeugodacus cucurbitae* Coquillett (Diptera:Tephritidae)

**Jilu V. Sajan · S. Subramanian · Kirti Sharma**

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**Abstract** *The melon fly, Zeugodacus cucurbitae* (Diptera:Tephritidae) is one of the devastating pests of most cucurbits in India and its management by application of synthetic insecticides remained unsuccessful. Tephritid fruit flies are reported to harbour symbiotic microbiota in their gut and ovipositor, providing them nutritional resources in the ecosystem. The current study was carried out to isolate the symbionts and to test their attraction potential for trapping the melon fly adults. From the molecular analysis of *16S rRNA* gene, 11 different species of bacteria were identified from gut and ovipositor of wild population of melon fruit fly. Among the identified bacteria, 7 species belonged to Enterobacteriaceae family, 2 species belonged to Bacillaceae family and one each belonged to Enterococcaceae and Flavobacteriaceae family. Among them *Enterococcus faecalis, Citrobacter sp., E. meningoseptica, Klebsiella oxytoca, Bacillus amyloliquefaciens* and *Bacillus sp.* were found only in ovipositor of the flies. Whereas, *Raoultella ornithinolytica* and *Klebsiella sp*. were found only in gut of the fruit flies. Two bacterial species, namely *K. pneumoniae* and *E. cloacae* were identified from both ovipositor and gut of wild flies. Five volatiles identified from these two bacteria were ammonia, dimethyl disulphide, methoxy phenyl oxime, 2, 2-dihydroxy-1-phenyl ethanone and butyl-2-methylpropyl ester of 1, 2-benzenedicarboxylic acid. The synthetic forms of identified volatiles and related compounds were tested for their potential to attract melon fruit fly. Ammonium hydroxide showed maximum attraction followed by iso amyl alcohol and dimethyl disulphide. Among the flies, 0–2 days old and 6–8 days old adults showed slightly higher attractancy towards the volatiles compared to 20–30 days old adults. Protein fed and protein starved adults showed maximum attractancy towards Ammonium hydroxide followed by Iso amyl alcohol. All the volatiles attracted more female flies compared to male flies.

**Keywords** Melon fruit fly · Symbiotic bacteria · Bacterial volatiles · Adults attraction · Adults trapping

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## Introduction

The melon fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae), one of the most important pests of cucurbitaceous vegetables (Sunil et al., 2016) was first reported from India in 1913 by Bezzi and considered it to be a native of Oriental region of Central Asia (Virgilio et al., 2010). This fruit fly pest is reported from more than 40 countries

including Afghanistan, Bangladesh, Bhutan, China, Iran, Pakistan and India infesting over 125 species of plants (Hadapad et al., 2016). This pest is reported to cause more than 50% losses in cucurbits and the losses can reach up to 100% if management practices are not applied efficiently (Dhillon et al., 2005). The synthetic insecticides being commonly applied, remained insufficient to manage this pest up to the acceptance level. The area-wide integrated pest management (AW-IPM) incorporating the sterile insect technique (SIT) has been reported to eradicate this pest from Southwestern Islands of Japan (Koyama et al., 2004). Since the SIT is inversely density dependent, it works at its best when applied after decreasing the density of target pest's population (Hendrichs & Robinson, 2009). The male annihilation technique incorporating the Cue lure do its share in population suppression by reducing the number of males in wild while baiting the females by protein baits also played an important role in decreasing the fruits infestation as a proportion of wild younger females are killed before infesting the fruits (Kakinohana et al., 1993). In addition to the established techniques of fruit flies population suppression, new/novel means of population suppression which can be incorporated as components of AW-IPM are much desired. The symbiotic bacteria or their volatiles has been considered potential agents for attracting adult fruit flies. Several studies have demonstrated that bacteria associated with flies showed attraction to flies (MacCollom et al., 1992; Martinez et al., 1994; Reddy et al., 2014; Robacker et al., 1991). Different bacteria produce a wide range of volatile organic compounds as by-products of different metabolic activities (Tait et al., 2013) such as alcohols, amines, ammonia, phenols, pyrazines, sulphur compounds, acids and ketones and many of them are reported to attract adults of fruit fly (Liscia et al., 2013). Robacker and Bartelt (1997) reported that volatile compounds identified from symbiotic bacteria from Mexican fruit fly, *Anastrepha ludens* (Loew) like, *Klebsiella pneumoniae* and *Citrobacter fruendii* showed better attractancy towards protein starved and sugar fed Mexican fruit flies.

In another study, Robacker et al. (1998) tested attractancy of 11 bacterial filtrates from four genera viz., *Enterobacter*, *Alcaligenes*, *Bacillus* and *Micrococcus* to *A. ludens* in laboratory bioassays. They demonstrated that different nitrogen containing chemicals and carboxylic acids were responsible for the attractancy of the bacterial filtrates to *A. ludens*. They isolated volatiles such as aliphatic amines, ammonia, imines, acetic acid and pyrazines from the bacterial filtrates. Epsky et al. (1998) studied attractancy of volatiles produced by symbiotic bacteria *E. agglomerans* to female flies of *Anastrepha suspensa* in laboratory condition. The most important volatiles produced by the bacterial culture were identified as 3-Methyl-1-butanol and ammonia and these chemicals were absent in sterilized Tryptic soy agar media.

As per the previous studies, both sexes of fruit flies are reported to be attracted to the odour produced by symbiotic bacteria (Hadapad et al., 2016). The odour is mostly due to the metabolites produced by bacteria. So identification of the odorous metabolites which are responsible for the attractancy may open a new area of pest management strategy against fruit flies. The current study focuses on bio-assaying the attractancy potential of volatiles produced by symbiotic bacteria associated with the melon fruit fly.

## Materials and methods

### Collection of test insect

Gourds infested with *Z. cucurbitae* were collected from vegetable field during April, 2015 from farmer's fields at Yamuna Bank (28.62° N, 77.27 ° E), New Delhi. Melon fly culture was established by rearing under laboratory conditions. Rearing was carried out at 27 ± 1 °C temperature and 70 ± 5% relative humidity with a natural photoperiod in the culture room. Pumpkin based artificial diet (containing pumpkin, yeast autolysate and methyl paraben) developed in our laboratory was used for oviposition (in small petriplates) and maggot rearing in laboratory condition. After oviposition, the petriplate with diet was kept in glass jars (20 × 15 cm), in which maggot rearing was carried out. The glass jars were filled with sterile sand up to 5 cm depth to facilitate the pupation. The jars were kept without any disturbance for adult emergence. On emergence, adults were transferred to rearing cages and provided with sugar, yeast autolysate and water.

## Isolation and identification of symbiotic bacteria from *Z. cucurbitae*

### *Dissection of gut and ovipositor*

The bacteria were isolated from the gut of male as well as gut and ovipositor of female adults of *Z. cucurbitae* using method described by Shi et al. (2012). Five adults of each sex were dissected and the gut was separated. For dissection of ovipositor, ten female flies were taken. Flies were cold anesthetized in refrigerator at 4 °C for 5 min. They were surface sterilized by immersing in 70% ethanol for 30 s followed by dipping in 0.25% sodium hypochlorite for 1 min and then washed thrice with sterilized distilled water (SDW) to remove the external contamination of microbes under aseptic condition. Wings and legs were removed and flies were fixed on sterilized wax plate. The flies were individually dissected on wax plates containing 3% saline under Stereomicroscope (Leica ES2) in laminar air flow. The dissected gut and ovipositor were separated from the flies and placed in eppendorf tubes containing 1 ml of 3% saline solution using a sterilized needle.

### Isolation and culturing of symbiotic bacteria

Both gut and ovipositor were homogenized separately using a homogenizer and dilutions ($10^{-1}$ to $10^{-6}$) were prepared. A small drop of homogenized samples were spread plated on petriplates containing tryptic soya agar (TSA) and nutrient agar (NA) media separately (Reddy et al., 2014; Jilu et al., 2018). Different plates were incubated at 30 ± 2 °C and 37 ± 2 °C for 24–48 h. Following incubation, morphologically distinct colonies were picked and streaked on to respective TSA and NA plates for growth. Pure bacterial isolates were obtained by repeated sub culturing of the isolates. Isolates were preserved in agar slants and in 50% glycerol.

### DNA extraction and PCR amplification of *16S rRNA* genes

Genomic DNA was extracted from 2 ml tryptic soya broth (TSB) and nutrient broth (NB) (Reddy et al., 2014) overnight cultures of individual bacterial isolates using modified CTAB method of DNA extraction. Genomic DNA was extracted using ZR Fugal/Bacteria DNA Micropep™ Kit (Zymo Research CA, USA). Genomic DNA obtained was visualized after electrophoresis in a 0.8% agarose gel in 1xTAE buffer to assess their integrity and then stored at −20 °C prior to PCR amplification.

PCR amplification of *16S rRNA* gene sequencing (Thaochan et al., 2010a) of the isolates was done using the universal bacterial primers 27F (5'-AGA GTTTGATYMTGGCTCAG-3′) and 1492R (5'-TAC CTTGTTAYGACTT-3′). The final reaction volume of 50 µl contained 2 µL each (2 mM) of forward and reverse primers, 25 µL of master mix, 19 µL of nuclease free water and 2 µL of (500 ng) template DNA. The PCR reactions carried out in a thermal cycler (Bio-Rad U.S.A) is as follows: Initial denaturation at 94 °C for 5 min followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min 40 s and final extension at 72 °C for 10 min. PCR products were resolved on 2% agarose gel stained with ethidium bromide and visualised under a gel documentation system. DNA sequencing of purified *16S rRNA* gene PCR product was carried out by out sourcing with SciGenom Labs Pvt. Ltd. Cochin, Kerala (India) using Sanger's dideoxynucleotide sequencing method and sequences obtained were compared with those in the GenBank database using the BLAST search algorithm (http://www.ncbi.nlm.nih.gov/). Sequences obtained were submitted to NCBI GenBank for and assigned accession numbers from KT895290 to KT895300. The nucleotide sequences were aligned using Clustal W2. Phylogenetic analysis was performed with program MEGA 6.0 (Molecular Evolutionary Genetics Analysis, Version 6.0) (Tamura et al., 2013) and phylogenetic trees were inferred using the neighbour-joining method (Yamvrias et al., 1970).

## Extraction and identification of volatiles from mass culture of bacteria

### *Detection of ammonia from bacterial cultures*

Two bacterial isolates *K. pneumoniae* and *Enterobacter cloacae* were used for the identification of volatiles as they showed maximum attractancy in the laboratory (Jilu et al., 2018). Low molecular weight compound like ammonia was detected from bacterial

cultures by traditional method (qualitative fuming method). For this, 1 L of NB media was prepared in a conical flask and inoculated with 100 µL of pure culture followed by incubation at 37 ± 1 °C for 72 h. After incubation, the cotton plug of conical flask was replaced with a glass hood with a glass tube attached at right angle to it. A petriplate with concentrated HCl was kept at the opening of the tube and presence of ammonia was detected by observing presence of white smoke above the petriplate. Similar set up was maintained by uninoculated media to confirm that the ammonia is not produced by the media.

Bacterial preparation for conducting GC/MS

The bacteria culturing and bioassays were performed following the method described by Martinez et al. (1994). Both *K. pneumoniae* and *E. cloacae* were inoculated on NA media in glass vials (which can be used directly on Varian PAL Autosampler' for sample collection) and incubated for 72 h at 37 °C. After incubation, the vials were plugged with teflon cap and sealed properly and used for conducting GC/MS.

Volatile sampling and GC/MS analysis of volatiles

A GC/MS instrument (Varian model 4000 GC/MS/MS) equipped with a 30-m VF-5 ms capillary column (Varian CP 8944), with an internal diameter of 0.25 mm and 0.25-µm film thickness was used (Gopal et al., 2015). Helium was used as carrier gas and gave a column head pressure of 10.5 psi and an average flux of 1 ml $min^{-1}$. An injector with split mode, with a temperature program of 200 °C for 2 min and then increased up to 265 °C at the rate of 20 °C $min^{-1}$ was used. The temperature program for the GC column consisted of a 10 min hold at 60 °C, then 5 °C $min^{-1}$ increase to 150 °C and a 3 min hold at 150 °C, followed by 10 °C $min^{-1}$ increase to 200 °C and a 5 min hold at 200 °C, then 20 °C $min^{-1}$ increase to 260 °C and a 1 min hold at 260 °C. The total run time was 45 min. Trap, manifold and transfer line temperature were set at 200, 60 and 270 °C respectively. The instrument was auto tuned. Ion trap tests and mass calibration were conducted weekly with perfluorotributylamine. The analyses were conducted with a filament delay of 2 min, and under automatic gain control with target values of 20,000 for GC/MS. Mass detector was in EI mode with mass range of 35 m/z to 245 m/z. A 'Varian PAL Autosampler' with 1 ml headspace syringe was used for sample collection. Syringe temperature and agitator temperature were 80 °C and 70 °C respectively. Sample (500 µL volume) was injected and incubated at 500 rpm for 5 min. Peaks obtained in the chromatogram were identified by comparing those with NIST library.

Attraction bioassay with bacterial volatiles in laboratory

An olfactometer cage developed in our laboratory (Reddy et al., 2014; Jilu et al., 2018) was used to evaluate the attractiveness of bacterial volatiles to melon fruit flies. The arms of the hub unit were revolved manually after every hour to ensure the orientation of flies towards the attractant source only. Different synthetic chemicals such as, Isoamyl alcohol, Isobutyl alcohol, Indole, Butyric acid, Ammonium hydroxide and dimethyl disulphide were used to test attractancy. We utilized commercially available synthetic chemicals which are closely related to the volatiles produced by bacteria as they are cheaper and easily accessible. Sterilized distilled water, sugar solution (10%) and yeast autolysate (source of protein) were used as control treatment to compare the attractancy. To test the attraction of *Z. cucurbitae* adults, 100 µL of each chemical and control was kept in the glass bulbs as such. After pouring these glass bulbs were sealed with lid at least for 2 h before the initiation of test. The bulbs with treatments were attached randomly to hub arms. Hub was placed at an equally lighted space. The experiment was carried out between 10.00 and 16.00 h under a combination of fluorescent and natural light. The different parameters studied were sex (male and female), age (0–2 days, 6–8 days and 20–30 days) and feeding status (protein-fed and protein-starved) of the adult flies. Fifty flies (sex ratio 1:1) for each treatment combinations were released into the cage 1 h before the start of experiments. After every one hour trapped flies were counted and total six observations was recorded. The average of 6 observation was taken.

Each experiment was replicated three times and data were analysed using SAS statistical software. The number of fruit flies oriented towards the treatment sources and entered in the adult bioassay cages was converted into percentages and compared by one-way analysis of variance (ANOVA), after the results

had been square-root transformed to meet the homogeneity of variances.

## Results

In total, 28 morphologically distinct colonies of bacteria were isolated, 14 colonies were from the dissected gut and 14 from the ovipositor of wild population of *Z. cucurbitae*. Eleven different species of bacteria were identified by *16S rRNA* gene sequencing analysis. Among the identified bacteria, 7 species belonged to Enterobacteriaceae family, 2 species belonged to Bacillaceae family and one each belonged to Enterococcaceae and Flavobacteriaceae family. Among the isolated bacteria, *Enterococcus faecalis, Citrobacter sp., Elizabethkingia meningoseptica, Klebsiella oxytoca, Bacillus amyloliquefaciens* and *Bacillus sp.* were found only in ovipositor of the flies. Whereas, *Raoultella ornithinolytica* and *Klebsiella sp*. were found only in gut of the fruit flies. Three bacterial species, namely *Klebsiella pneumoniae, Enterobacter sp.* and *Enterobacter cloacae* were identified from both ovipositor and gut of wild flies (Table 1). The phylogenetic trees represents the phylogenetic relationship of different bacteria found in the gut and ovipositor of wild flies of *Z. cucurbitae* (Figs. 1 and 2).

Two bacterial cultures namely *K. pneumoniae* and *E. cloacae* produced ammonia and was detected with the presence of white fumes in the traditional method of detection. GC/MS analysis of volatiles from treated NA (inoculated with *K. pneumoniae* and *E. cloacae*) and blank NA media are presented in Fig. 3. Volatiles identified include dimethyl disulphide (2.747 min, m/z = 94), methoxy phenyl oxime (7.179 min, m/z = 151), 2,2-dihydroxy-1-phenyl ethanone {35.992 min,

**Table 1** Identification of bacteria isolated from ovipositor and gut of wild adults of *Z. cucurbitae* based on *16S rRNA* gene sequencing

| Sl. No. | Accession number | Nearest neighbour Species | Family | Length of *16S rRNA* gene sequenced (bp) | Similarity (%) with nearest neighbour in NCBI database |
|---|---|---|---|---|---|
| From ovipositor | | | | | |
| 1. | KT895290 | *Enterococcus faecalis* | Enterococcaceae | 1460 | 99 |
| 2. | KT895291 | *Klebsiella pneumoniae* | Enterobacteriaceae | 1448 | 99 |
| 3. | KT895292 | *Citrobacter sp.* | Enterobacteriaceae | 1441 | 99 |
| 4. | KT895293 | *Elizabethkingia meningoseptica* | Flavobacteriaceae | 1112 | 85 |
| 5. | KT895294 | *Klebsiella oxytoca* | Enterobacteriaceae | 1444 | 99 |
| 6. | KT895295 | *Bacillus amyloliquefaciens* | Bacillaceae | 1110 | 93 |
| 7. | KT895296 | *Bacillus sp.* | Bacillaceae | 1458 | 98 |
| From gut | | | | | |
| 8. | KT895297 | *Raoultella ornithinolytica* | Enterobacteriaceae | 1446 | 98 |
| 9. | KT895298 | *Enterobacter sp.* | Enterobacteriaceae | 1441 | 99 |
| 10. | KT895299 | *Enterobacter cloacae* | Enterobacteriaceae | 1440 | 98 |
| 11. | KT895300 | *Klebsiella sp.* | Enterobacteriaceae | 1443 | 99 |

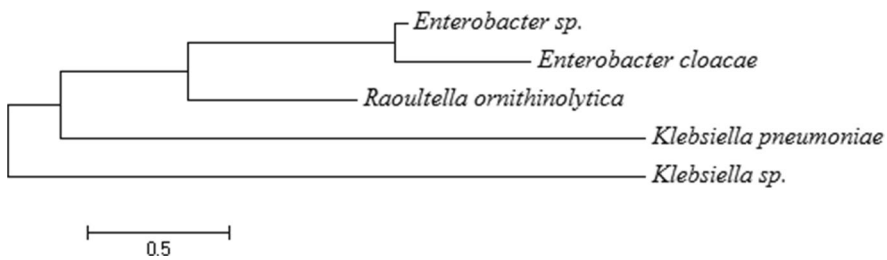



**Fig. 1** Phylogenetic tree constructed using *16S rRNA* gene sequences showing relationship between gut bacterial isolates of wild (collected from Yamuna Bank, New Delhi) adult flies of *Z. cucurbitae*

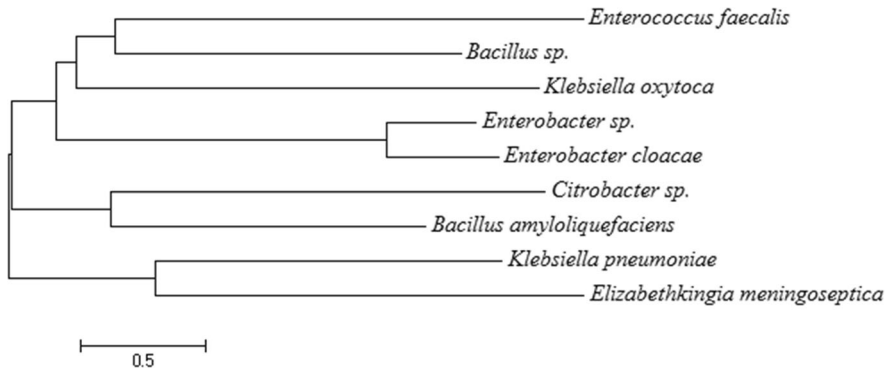



**Fig. 2** Phylogenetic tree constructed using *16S rRNA* gene sequences showing relationship between ovipositor bacterial isolates of wild (collected from Yamuna Bank, New Delhi) adult flies of *Z. cucurbitae*

base peak = 105 [152(m/z)-$H_2O$(18)-CO(28)]} and butyl-2-methylpropyl ester of 1,2-benzenedicarboxylic acid {36.495 min, base peak = 149 [278(m/z)-C4H9(57)-C3H7(43)-CO(28)]}.

Statistical analysis of the attractancy data from olfactory cage bioassay showed that 0–2 days old and 6–8 days old adults showed slightly higher attractancy towards the volatiles compared to 20–30 days old adults. Protein fed 0–2 days old adults showed maximum attractancy towards Ammonium hydroxide (3.86 ± 0.39 flies) followed by Iso amyl alcohol (3.56 ± 0.71 flies). In case of 6–8 days old protein fed adults mean attractancy towards Iso amyl alcohol and Ammonium hydroxide was (3.74 ± 0.27 flies) and (3.72 ± 0.54 flies) respectively. Ammonium hydroxide (3.70 ± 0.69 flies) showed maximum attractancy followed by Iso amyl alcohol (3.51 ± 0.75 flies) in case of 20–30 days old protein fed adults. Protein starved 0–2 days old adults were attracted towards Ammonium hydroxide (3.77 ± 0.39 flies) followed by Iso amyl alcohol (3.45 ± 0.37 flies). Ammonium hydroxide (3.79 ± 0.08 flies) attracted significantly higher number of protein starved 6–8 days old *Z. cucurbitae* adults followed by Iso amyl alcohol (3.51 ± 0.22 flies) and Dimethyl disulphide (3.16 ± 0.16 flies). Ammonium hydroxide and Iso amyl alcohol showed high attractancy potential with a mean adult catch of (3.49 ± 0.45 flies) in case of protein starved 20–30 days old fruit flies. All the volatiles showed significantly higher attractancy than the control treatments. Protein starved flies were more attracted towards yeast autolysate compared to protein fed adults. More number of female flies was attracted towards the volatiles compared to male flies (Fig. 4).

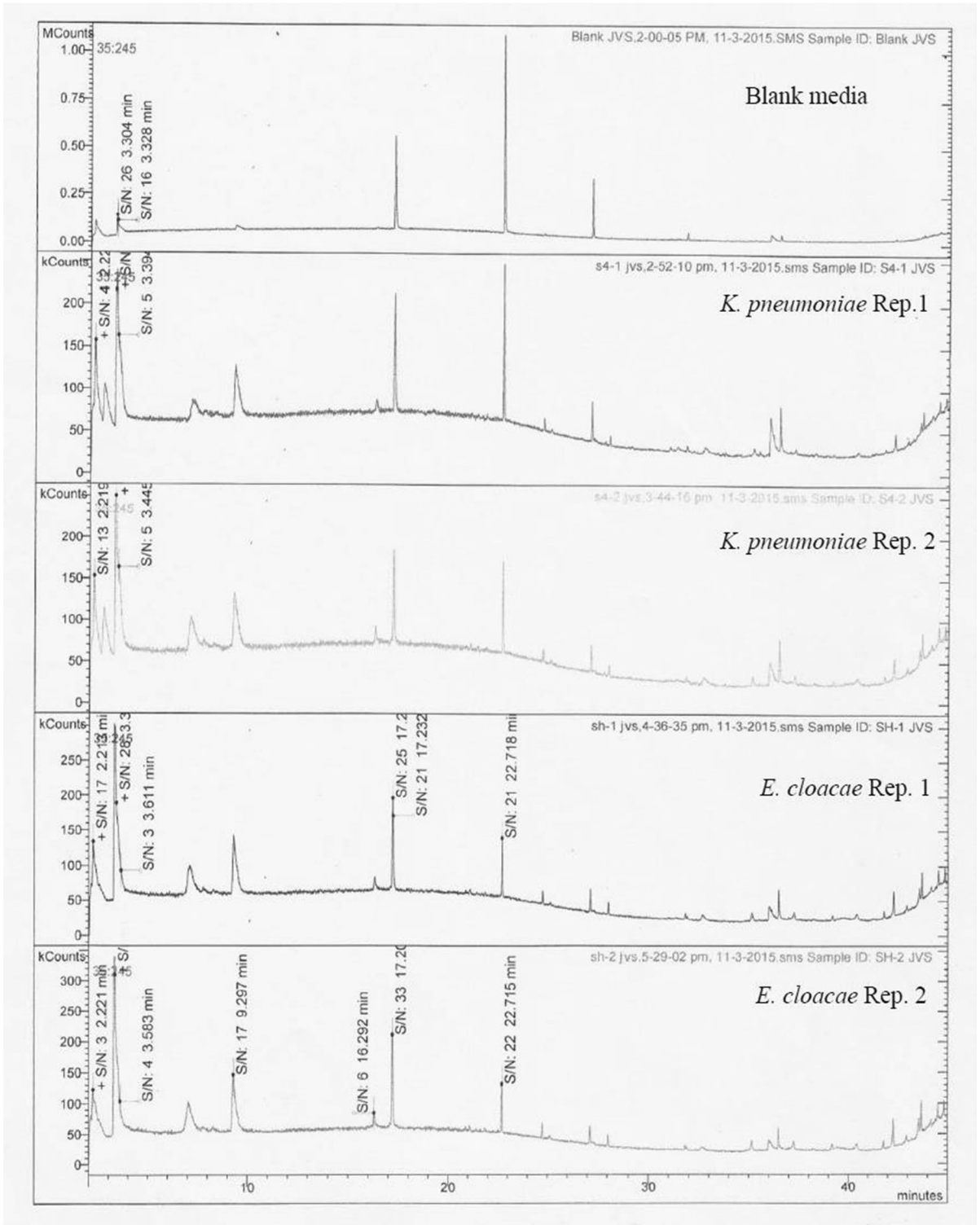
## Discussion

In the current study, among the isolated bacteria, 7 species belonged to Enterobacteriaceae family, 2 species belonged to Bacillaceae family and one each belonged to Enterococcaceae and Flavobacteriaceae family. Our results are in line with the previous findings by Sharma and Reddy (2012) and Wang et al. (2011) who reported Tephritid symbiotic bacteria mostly belong to family Enterobacteriaceae and two species, viz. *Klebsiella* and *Enterobacter* as predominant ones. Yamvrias et al. (1970) identified the bacteria from the oesophageal diverticulum of field collected adults of *Dacus* (*Bactrocera*) *oleae* (Gmelin) and found most of them are gram negative and belong to Pseudomonadaceae and Enterobacteriaceae. This result also supports the current findings. Cultivable bacteria consisting of Enterobacteriaceae, Bacillaceae and Enterococccaceae were reported from the gut of *Bactrocera dorsalis* (Wang et al., 2014). The identified species from *Z. cucurbitae* mainly belonged to Enterobacteriaceae, Bacillaceae and Enterococccaceae in our study also. Similar to our results, different bacterial species were isolated and identified from the digestive tract of *Bactrocera*, *Anastrepha, Dacus, Rhagoletis* and *Ceratitis* species and they mostly belonged to family Enterobacteriaceae, Bacillaceae, Vibrionaceae, Pseudomonadaceae, Staphylococcaceae and Micrococcaceae (Hadapad et al., 2016; Wang et al., 2011; Wang et al., 2014). We got maximum diversity of bacteria belonging to Enterobacteriaceae family. The *Citrobacter sp.* was identified by Hadapad et al. (2016) from *Z. cucurbitae. Klebsiella oxytoca* was identified from *Ceratitis capitata* (Wiedemann) (Marchini et al., 2002), *B. tau* (Prabhakar et al., 2009), *B. cucuminata* and *B. tryoni*

(Thaochan et al., 2010b), *Z. cucurbitae* (Thaochan et al., 2010a) *B. dorsalis* (Shi et al., 2012) and *B. zonata* (Reddy et al., 2014). Similarly, *Bacillus sp.* was reported from *B. zonata* (Reddy et al., 2014) and *Z. cucurbitae* (Hadapad et al., 2016). Presence of *Klebsiella sp.* in the gut of fruit flies was previously reported by Wang et al. (2011) from *B. dorsalis*, Sharma and Reddy (2012) from *B. zonata*, Yuval et al. (2013) from Mediterranean fruit fly and Pramanik et al. (2014) from *B. dorsalis*. Several earlier studies have reported the presence of *K. pneumoniae* as a predominant gut symbiont in different species of fruit flies like *A. ludens* (Kuzina et al., 2001), *Z. cucurbitae* (Thaochan et al., 2010a), *B. dorsalis* (Shi et al., 2012) and *B. zonata* (Reddy et al., 2014). *Enterobacter sp.* is also one of the most abundant species of bacteria reported from Mexican fruit fly (Kuzina et al., 2001), *C. capitate* (Marchini et al., 2002; Yuval et al., 2013), *B. dorsalis* (Wang et al., 2011; Pramanik et al., 2014) and *B. zonata* (Reddy et al., 2014). Many other species of bacteria were also reported to be abundant in the gut of fruit flies such as *B. cucuminata, B. tryoni* (Thaochan et al., 2010b), *Z. cucurbitae* (Jilu et al., 2018; Thaochan et al., 2010a) and *B. zonata* (Reddy et al., 2014). Our studies have also established the presence of *K. pneumoniae*, *E. cloacae* and *Klebsiella sp*., as the most abundant bacterial symbiont detected in the gut and ovipositor of *Z. cucurbitae.* In our study we have isolated bacteria from ovipositor because of the presence of bacteria on the egg surface.

During our previous laboratory studies (Jilu et al., 2018) we identified two bacterial cultures namely *K. pneumoniae* and *E. cloacae* has potential attractant property (Reddy et al., 2014) towards the melon fruit flies. Interestingly these two bacteria were the only species of bacteria we could able to isolate from both gut and ovipositor of melon fruit flies. Certain components of bacterial odour have been reported to modulate the behaviour of fruit flies either as feeding or as ovipositional stimulants (Drew & Lloyd, 1987; Lauzon et al., 1998) and some of these components have been used in pest management in the form of baits or traps. The large scale production of bacterial culture and their maintenance is a tedious process and when used in traps there are chances of contamination by other micro-organisms which may hamper the viability, durability and stability of attractant traps. So we tried to identify the component responsible for the attraction of the flies. As, the odour of bacterial culture is mainly derived from the volatile compounds produced by the bacterial species, the identification of these volatile compounds and their utilization in testing attractancy of the flies instead of bacterial cultures sound reasonable. *Klebsiella pneumoniae* and *E. cloacae* showed presence of ammonia in our studies. Epsky et al. (1998) also detected ammonia from *E. agglomerans* isolated from *A. suspensa.* Similarly, Robacker et al. (1998) detected ammonia from symbiotic bacterial isolates of Mexican fruit fly. Further, in our study, ammonium hydroxide showed the highest attractancy of the flies. So the presence of ammonia in these cultures might largely contributed to their attractancy potential.

During GC/MS analysis of different treatments, some peaks (namely at 3.3, 9.29, 17.2, 22.7, 27.0, 32.0 min) were observed in blank media. Therefore, these peaks were not considered for volatile evolved in the treated media. The additional peaks present in the treated NA media were identified as dimethyl disulphide, methoxy phenyl oxime, 2,2-dihydroxy-1-phenyl ethanone and butyl-2-methylpropyl ester of 1,2-benzenedicarboxylic acid. In a study conducted by Robacker and Bartelt (1997), they identified dimethyl disulphide from *K. pneumoniae.* An experiment by Stotzky and Schenck (1976) proved presence of dimethyl disulphide in *Clostridium sp.* The extraction of these volatiles from bacterial cultures and their utilization for trapping fruit flies seemed not feasible practically. So in this study, we decided to utilize commercially available chemicals which are related to the volatiles reported by different workers in attractancy bioassays. Seven volatile compounds were compared to identify the compound with most attractancy to different age groups of melon fruit flies. We observed that 0–2 days old and 6–8 days old adults showed slightly higher attractancy towards the volatiles compared to 20–30 days old adults. Robacker and Garcia (1993) observed in a laboratory bioassay with Mexican fruit flies that the attraction to the bacteria started when the flies were 1 day old, then reached peak when flies were 5–7 days old, then declined steadily as flies aged further. Protein fed and protein starved adults showed maximum attractancy towards Ammonium hydroxide followed by Iso amyl alcohol. Once the flies reached sexual maturity, their foraging for protein is reduced and oviposition activities are enhanced, therefore,


Blank media
K. pneumoniae Rep.1
K. pneumoniae Rep. 2
E. cloacae Rep. 1
E. cloacae Rep. 2
minutes

◂**Fig. 3** GC/MS Chromatogram plots of different treatments (peaks at 2.747 min - dimethyl disulphide, 7.179 min - methoxy phenyl oxime, 35.992 min - 2,2-dihydroxy-1-phenyl ethanone and 36.495 min - butyl-2-methylpropyl ester of 1,2-benzenedicarboxylic acid). (AH: Ammonium hydroxide, BA: Butyric acid, DDS: Dimethyl disulfide, Ind: Indole, IAA: Iso amyl alcohol, IBA: Iso butyl alcohol)

least response was observed after 20 days. Robacker and Bartelt (1997) while evaluating the volatile compounds from *K. pneumoniae* and *C. fruendii* for their attractancy against Mexican fruit fly under laboratory conditions, observed that all chemicals containing protonizable nitrogen, except 2-methylpyrazine, were attractive. Synthetic mixtures of ammonia, trimethylamine, 1- pyrroline, 3- methylbutamine, pyrazine, 2,3,4,5-tetrahydropyridine, 2,5-dimethylpyrazine and trimethylpyrazine in concentrations similar to those in filtrates of the two bacteria were 73–87% as attractive as bacterial filtrates. Volatiles such as ammonia, methylamine, 3-methylbutanamine, 1-pyrroline, 2, 3, 4, 5-tetrahydropyridine and several pyrazines from endosymbionts of *A. ludens* like *K. pneumoniae* and *C. freundii* were identified as attractants to the fruit flies (Robacker et al., 1997). Volatiles like 3-hydroxy-2- butanone, 2-phenylethanol, ammonia, indole and trimethyl pyrazine from two strains of *E. agglomerans* were also reported to attract sugar-fed adults of *A. ludens* (Robacker & Lauzon, 2002; Robacker et al., 2004). The increased attractancy of melon fruit flies towards Ammonium hydroxide is in agreement with the findings of several earlier works who demonstrated the attractancy potential of Ammonia against different species of fruit flies. In another study, Hadapad et al. (2016) reported volatiles i.e. 3-methyl-1-butanol, 2-phenylethanol, butyl isocyanatoacetate, 2-methyl-1-propanol and 3-hydroxy-2-butanone emitted by endosymbionts of *Z. cucurbitae* like *K. oxytoca* and *C. freundii* were attractant sources of melon fruit flies. All the volatiles showed significantly higher attractancy than the control treatments in this study.

Protein starved flies were found to be more attracted towards yeast autolysate compared to protein fed adults in our various experiments. Robacker and Garcia (1993) reported a similar result as sugar-fed, protein-deprived flies were more attracted towards the bacterial odour compared to protein fed adults. Bacterial odour was also attractive to sugar and protein fed flies. Reddy et al. (2014) reported a significant difference in the attractancy towards bacteria between the protein fed and protein starved flies of different age groups. In all the experiments more female flies were attracted to the volatiles compared to male flies.

## Summary

In the current study we identified 11 species of culturable endosymbiotic bacteria associated with gut and ovipositor of melon fruit fly adults. By identifying

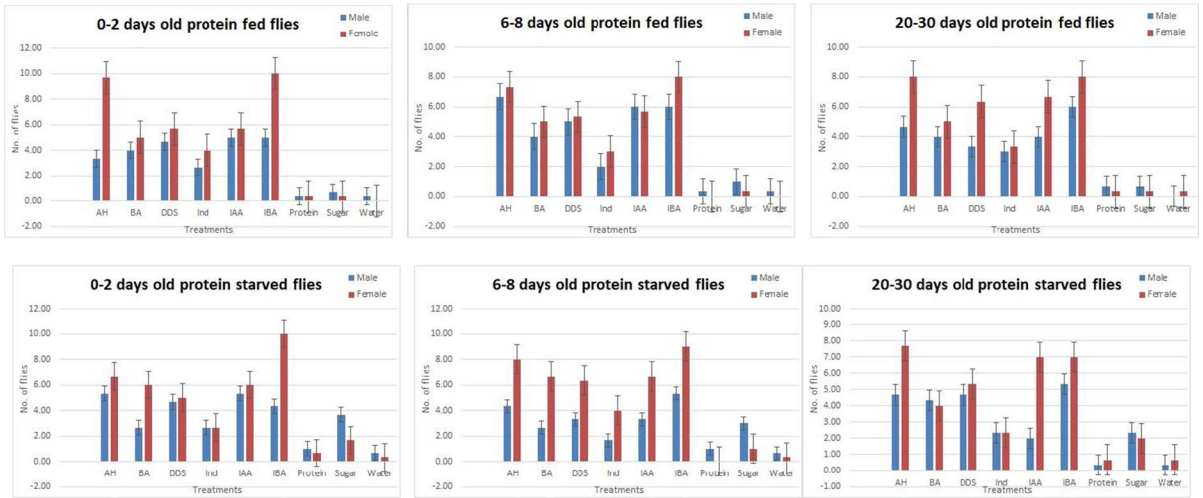



**Fig. 4** Attractancy of different sex and age groups of melon fruit flies to bacterial volatiles

the volatiles from endosymbionts and experimentally proving the attractancy potential of these volatiles or similar synthetic compounds towards the adults of melon fruit fly, provided the scientific evidence of potential use of these compounds for eco-friendly management of this devastating pest.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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