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High Taxonomic and Functional Diversity of Bacterial Communities Associated with Melon Fly, *Zeugodacus cucurbitae* (Diptera: Tephritidae)

Jaipal S. Choudhary[1] · Naiyar Naaz[1,2] · Chandra S. Prabhakar[1,3] · Bikash Das[1] · Arun K. Singh[1] · B. P. Bhatt[4]

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Abstract

The next generation sequencing (NGS) approach has facilitated the investigations of gut microbiota with high throughput and resolution. The present study was focused on the taxonomic and functional characterization of bacterial community associated with different developmental stages of melon fly, *Zeugodacus cucurbitae* using 16S ribosomal RNA (rRNA) gene amplicons metagenomics. *Z. cucurbitae* is considered an invasive and most staid polyphagous pest of cucurbitaceous and other related crops. The taxonomic analysis of highly variable V3–V4 region of bacterial 16S rRNA gene sequencing indicated that the bacterial community associated with *Z. cucurbitae* consists of a total of 23 bacterial phyla (including unclassified and unassigned bacteria), comprising 32 classes, 69 orders, 99 families and 130 genera. Proteobacteria, Firmicutes, Actinobacteria and Tenericutes were dominant phyla of which family, Enterobacteriaceae was the most abundant in the larval and adult female stages, whereas Mycoplasmataceae was the dominant in the pupal stage. In larval stages of *Z. cucurbitae*, genus *Providencia* and *Comamonas* were the most abundant. However, genus *Candidatus*-Bacilloplasma and *Klebsiella* were the most dominant in pupae and adult females of *Z. cucurbitae*, respectively. PICRUSt analysis conducted for prediction of metabolic activities revealed that associated microbiota were involved in membrane transport, carbohydrate metabolism, amino acid metabolism, energy metabolism, replication and repair processes as well as cellular processes and signalling. The higher number of OTUs was annotated for phosphoglycerate mutase and transketolase in adult females followed by larval stages, which may support the digestive function of the microbiota in larvae and adult females. Our findings provide insights about the high variation in microbiota across developmental stages and basis for microbiota-based management strategies of fruit flies.

Introduction

Fruit flies (Diptera: Tephritidae) are the most invasive and serious pests of many horticultural crops. The melon fly, *Zeugodacus cucurbitae* (Coquillett, 1899) earlier known as *Bactrocera cucurbitae* is one among them. It is a polyphagous insect pest of cucurbitaceous and other related agriculturally important crops. It is native of Central Asia and further established into most of the Asia, several countries of Africa, South Pacific islands of South America and islands of Oceania and Hawaii [1]. Due to its wide adaptability, high fecundity and establishment capacity into new environment, *Z. cucurbitae* needs special approaches in pest management strategies.

Insects harbour a range of microorganisms in their gut, haemocoel, on the exoskeleton and within the insect cells [2]. These microorganisms play significant role in providing specific nutrients such as amino acids and vitamins, or digestive enzymes, activation of immunity against deleterious

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✉ Jaipal S. Choudhary
choudhary.jaipal@gmail.com

[1] Farming Systems Research Centre for Hill and Plateau Region, ICAR-RCER, Plandu, Ranchi, Jharkhand 834 010, India

[2] University Department of Botany, Ranchi University, Morabadi, Ranchi, Jharkhand 843 008, India

[3] Department of Entomology, Veer Kunwar Singh College of Agriculture, Dumraon (Bihar Agricultural University, Sabour), Buxar, Bihar 802 136, India

[4] ICAR-Research Complex for Eastern Region, ICAR Parisar, P. O. Bihar Veterinary College, Patna, Bihar 800 014, India

invaders, enabling their hosts to tolerate temperatures extremities, pathogen defence, detoxification of plant toxic secondary metabolites and synthesis of semiochemicals—source of cues and signals [3–6]. The composition and reorganization of insect associated microorganisms changes with change of host [6, 7], geographical regions and associated environment [8–10], strains and even their ontological stages [7, 11–13]. Insect associated bacterial communities have been explored as role of mating incompatibility, feeding behaviour modulation, and ovipositional stimulants in pest management programmes in the form of traps or baits [10, 14, 15].

Even if *Z. cucurbitae* is an agriculturally important insect species, limited studies on microbiota associated with it have been done by several researchers and most of them restricted in culture-dependent techniques or molecular approaches with low resolution [16, 17]. Till date, high throughput sequencing has been conducted for profiling the bacterial community associated with trap-caught adult male flies of *Z. cucurbitae* collected from three different geographic regions [18] as well as wild and mass-reared mature and newly emerged adults of *Z. cucurbitae* [19]. To the best of our knowledge, microbiota compositions of different development stages of *Z. cucurbitae* are not available. Information on microbiota associated with developmental stages is also limited for other *Zeugodacus* fruit fly species except *Zeugodacus tau* [20]. Thus, in the present study we report for the first time on bacterial communities associated with different developmental stages (1st instar, 3rd instar, pupa and adult female) of *Z. cucurbitae* using 16S rRNA gene metabarcoding on the Illumina HiSeq platform [7, 21]. We also predicted the functional metabolic activities of bacterial community associated with developmental stages of *Z. cucurbitae.* The present study results will provide data on bacterial diversity and relative abundance of microbiota associated with different developmental stages and their transmission from immature stages to adults of *Z. cucurbitae*.

Methods

Sampling of Different Stages of *Z. cucurbitae*

For sampling of different stages of *Z. cucurbitae,* cucumber, *Cucumis sativus* L. (Violales: Cucurbitaceae) infested by fruit flies were collected from Research farm of ICAR-RCER, Farming Systems Research Centre for Hill and Plateau Region, Ranchi, India (23° 45′N latitude, 85° 30′ E longitude, elevation 620 m AMSL) during May, 2018 and brought to the laboratory for further sampling. The infested cucumber fruits were kept on 5 cm thick sterile layer of sand inside at the bottom of insect rearing cages (30 × 30 × 30 cm size) for rearing of fruit flies in the laboratory with 25 ± 1 °C temperature; 65 ± 5% RH and 12L: 12D photoperiod. Newly emerged adult male and female flies of *Z. cucurbitae* were placed in Perspex cages (Bio-agent acrylic cage of Rescholar Equipment, India) (size: 30 × 30 × 30 cm) provided with sterilized artificial diet (semi-solid mixture of sugar and protein hydrolysate in 3:1 ratio) and water soaked cotton as source of water. After 10 days of adult emergence, fresh uninfested cucumber fruits were placed in the cages for oviposition. After hatching, 1st instar larvae were directly collected by cutting the cucumber infested by *Z. cucurbitae*. For rest of the stages collection, same rearing protocol was followed. The late 3rd instar was collected from larvae jumped out of infested cucumber for pupation. To obtain pupal sample, 3rd instar was left in the sand for pupation and furthermore, some pupae were left in the sand for emergence of adult stage flies. Five days aged adult females based on presence of ovipositor were collected to study the adult females associated microbiota. Each stage sample (1st instar, late 3rd instar, pupae and adult females) were separately placed into tubes and stored in − 80 °C for further DNA extraction.

DNA Extraction, Targeted V3–V4 Region Amplification and Sequencing

After collection, samples of different stages of *Z. cucurbitae* were surface sterilized with 0.1% mercuric chloride solution for 60 s followed by washing with sterilized distilled H_2O to remove traces of chemical. DNA extraction was performed with individual specimen of different stages using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The quantification of the DNA was subsequently done by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Isolated DNA from five individual specimens of each stages were pooled together to make one sample for each developmental stage of *Z. cucurbitae*. For characterization of bacterial community V3–V4 hypervariable region were amplified using primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACNVGGG-TATCTAATCC-3′). Library preparation was done with NEBNext Ultra II DNA Library Preparation Kit (New England BioLabs Inc.) following the manufacturer's protocol using NEBNextQ5 Hot HiFi PCR Master Mix (New England BioLabs, France) with PCR conditions of 30 s of initial denaturation at 98 °C, followed by 12 cycles of 10 s denaturation at 98 °C, 75 s annealing/extension at 65 °C and 2 min final extension additionally at 72 °C and the NEBNext Oligos Kit (2 × 250 bp). The sequencing was performed using Illumina HiSeq System (HiSeq Rapid SBS Kits v2) (2 × 250 nt paired-end reads) at AgriGenome Labs, Cochin, Kerala, following the manufacturer's run protocols (Illumina, Inc., San Diego, CA, USA).

Assembly, Annotation of Metagenomic Sequences and Statistical Analysis

Raw sequences obtained for different developmental stages were quality checked for different parameters viz., base quality parameters, base composition distribution and GC distribution using the FastQC software [22]. The raw sequences were processed and analysed using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) pipeline [23]. Paired Illumina reads were quality trimmed and joined using FLASH program (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/) [24] to obtain the consensus sequences after removal of the low quality read. Chimeric sequences were also removed using the UCHIME algorithm implemented in the tool VSEARCH, version 1.7.0 [25]. The sequence reads from all samples were pooled and clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity at 97% using Uclust program, version 1.2.22 [26] in QIIME (version 1.9.1). The taxonomy assigned to the sequences with reference to the Silva release 13.5 database (reference126) using PyNAST as the taxonomy assignment tool [27]. Biological Observation Matrix (BIOM) file was used for all the downstream analyses. The diversity analysis including Chao1, Ace, Shannon, Simpson and Fischer indices as well as heatmap of relative abundance were calculated using MicrobiomeAnalyst web platform (http://www.microbiomeanalyst.ca) with Bray–curtis distance [28]. The unique and shared OTUs across the developmental stages of *Z. cucurbitae* were represented with Venn diagram computed using the VennDiagram package in R 3.6.2 environment (http://www.R-project.org/). BLAST search was further carried out on NCBI nucleotide collection (nr/nt) using the BLASTn algorithm to obtain information on taxonomic identity of shared OTUs. To determine the average dissimilarities in bacterial community structure between different developmental stages of *Z. cucurbitae* and to assess which phylum was responsible for the observed differences similarity percentage (SIMPER) analysis was conducted in the PAST 3.25 software package and difference in bacterial community structure was tested by the χ^2 test [29]. The metabolic functions were predicted from the 16S rRNA sequences data using PICRUSt v. 1.1.4 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved State) software against the functional database of KEGG orthology [30]. Final sequence read achieves (SRAs) were deposited in the NCBI SRA database under the Bioproject accession ID PRJNA587221: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA587221.

Results

Description of 16S rRNA Gene Sequencing Reads

The highly variable V3–V4 region of bacterial 16S rRNA gene was sequenced on the Illumina HiSeq platform for pooled DNA extract from five individual specimens of four different developmental stages of *Z. cucurbitae*. A total of 950,166 of 16S rRNA raw sequence reads were generated from different developmental stages. After high-quality filtering and chimera removal from the raw sequences, the demultiplexed paired-end reads for each developmental stage of *Z. cucurbitae* ranged from 126,989 to 230,046 for adult female stage (CF) to pupal stage (CP), respectively.

Analysis of operational taxonomic units (OTUs) of bacterial community suggested that pupal stage harbours highest number of OTUs (CP: 1979) followed by the adult female stage (CF: 992). The variation in number of OTUs across the developmental stages indicates the complexity of stage-associated bacterial community of *Z. cucurbitae* (Table 1). Relationship between OTUs depicted through Venn diagram displaying only 15 shared OTUs among all studied developmental stages of *Z. cucurbitae* at 97% similarity cut-off (Fig. 1a). The core microbiota associated with various developmental stages of *Z. cucurbitae* were mainly belongs to phyla Proteobacteria (53.33%) and Tenericutes (33.33%) (Fig. 1b). The most abundant shared OTUs was represented by members of family Enterobacteriaceae (Supplementary file 1) with a BLAST search showing *Providencia vermicola* (accession number MN744552) in larval stages and *Klebsiella* sp. (accession number MN712475) in adult stages (Table 2). Enterobacteriaceae comprised 73.13% and 45.53%

Table 1 Summary of the 16 s rRNA sequencing data of microbiota associated with different development stages of *Zeugodacus cucurbitae*

ID	No. of bacterial reads	Pre-processed consensus sequences	Total consensus sequences	No. of observed OTUs[a]	GC content (%)	Avg. length (nt)
CL1	260,851	170,721	170,774	702	53.39	450
CL3	234,932	152,147	152,227	706	53.13	450
CP	261,872	230,046	230,238	1979	50.07	450
CF	192,511	126,989	126,994	992	55.18	450
Total	950,166	679,903		3738		

[a]OTUs (operational taxonomic units) at the 97% sequence similarity cut-off; CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

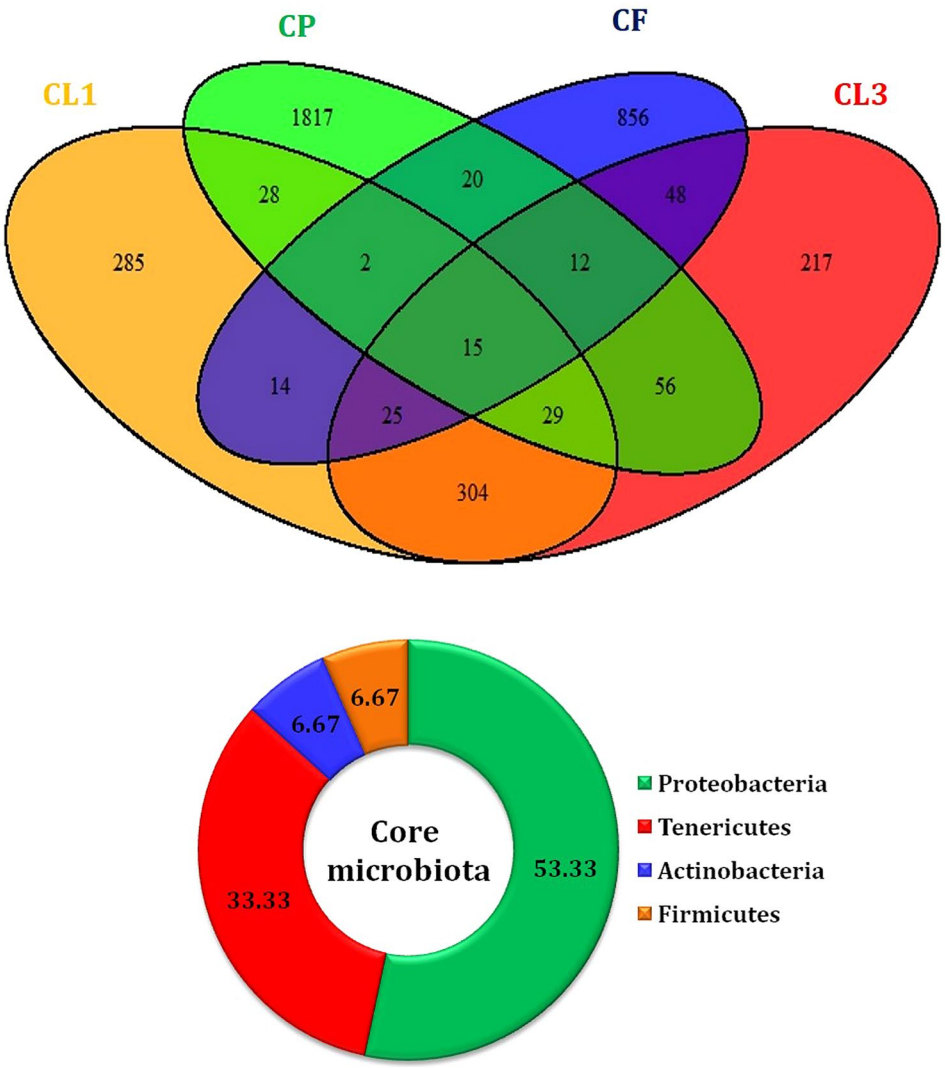

Fig. 1 OTUs (Operational Taxonomic Units) analysis between different developmental stages of *Zeugodacus cucurbitae* at 97% similarity **a** Venn diagram showing unique and shared OTUs, of which 15 OTUs shared between all developmental stages. **b** Percentage distribution of common shared OTUs (15 OTU) between all developmental stages at phylum level. CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

of bacteria present in first and second instar, respectively and 52.07% in adults. However, the shared OTU abundant in pupa belonged to genus *Candidatus*-Bacilloplasma of Mycoplasmataceae family. A BLAST search using Blastn program revealed the uncultured bacterium clone (accession number KF329419) as the closest match in pupal stage (Table 2). The diversity of bacterial community varied across the developmental stages of *Z. cucurbitae* as suggested by diversity indices (Table 3). The extensive taxonomic analyses of all developmental stages are available as supplementary data shown in excel file (supplementary file 1) and also as sunburst charts (Supplementary file 2).

Taxonomic Composition of Microbiota Associated with Different Developmental Stage

The taxonomic analysis of associated microbiota revealed that different stages of *Z. cucurbitae* consists of total 23 bacterial phyla (including unclassified and unassigned bacteria), comprising 32 classes, 69 orders, 99 families and 130 genera. Comparison among taxa showed that each developmental stage of *Z. cucurbitae* have distinct bacterial community (Fig. 2). Similarity analysis of bacterial community based on UPGMA clustering among different developmental stages showed that changes in bacterial community across developmental stage is not affected by preceding stages of *Z. cucurbitae* development. The bacterial community profile of 1st instar and adult were most similar, whereas bacterial community associated with pupae was least shared to those associated with other developmental stages. In pupal stage, bacterial community was highly divergent and forms the out group due to the exceptionally high dominance of phylum Tenericutes with respect to other developmental stages (Fig. 3). Only four phyla, Proteobacteria, Firmicutes, Actinobacteria and Tenericutes were dominant and shared among all the different developmental stages. Proteobacteria was the most predominant phylum in the larval and adult stages (mean ranged from 88.12 to 99.68% of total),

Table 2 Abundance of core microbiota across development stages of *Zeugodacus cucurbitae*, expressed as % of total in each life stage

OTU	Best match gene (Accession Number)	Best match gene %	Best match gene ID	Phylum	Order	Class	Family	CL1	CL3	CP	CF
OTU 30	MN744552	100	*Providencia vermicola*	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	73.133	45.534	0.023	0.013
OTU 102	MN657413	100	*Ochrobactrum ciceri*	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	0.011	0.009	0.001	1.213
OTU 449	CP045456	99	*Brevundimonas* sp.	Proteobacteria	Alphaproteobacteria	Caulobacteriales	Caulobacteraceae	0.005	0.004	0.100	0.003
OTU 1296	MN104669	98	*Klebsiella spallanzanii*	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	0.056	0.138	0.008	0.204
OTU 1348	GU293191	98	Uncultured bacterium	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	0.007	0.002	0.714	0.003
OTU 1643	MN709366	100	*Acinetobacter bereziniae*	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	0.483	1.177	0.012	0.009
OTU 1816	LC378890	99	Uncultured *Enterobacter* sp.	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	0.008	0.026	0.001	0.073
OTU 2204	KF328917	98	Uncultured intestinal bacterium	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	0.003	0.002	0.582	0.002
OTU 2681	MK825540	100	*Streptomyces puniceus*	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	0.001	0.002	0.012	0.007
OTU 2844	CP046123	100	*Enterococcus casseliflavus*	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	0.134	1.085	0.018	0.001
OTU 3170	KF329419	100	Uncultured bacterium	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	0.152	0.417	51.125	0.065
OTU 3400	KP950641	96	Uncultured intestinal bacterium	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	0.003	0.018	1.059	0.004
OTU 3513	MN104668	98	*Klebsiella* sp.	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	0.008	0.036	0.001	0.123
OTU 3678	KF329391	97	Uncultured intestinal bacterium	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	0.002	0.009	1.043	0.001
OTU 3697	MN712475	100	*Klebsiella* sp.	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	2.052	3.776	0.017	52.067

CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

Table 3 Diversity Indices of bacterial community in different developmental stages of *Zeugodacus cucurbitae*

ID	Shannon index	Simpson index	Chao 1	Ace	Fischer
CL1	1.17	0.42	358.32 ± 6.50	359.32 ± 8.72	44.05
CL3	2.07	0.72	438.25 ± 8.05	440.13 ± 9.61	56.56
CP	1.61	0.47	143.67 ± 2.20	144.25 ± 5.01	16.24
CF	1.96	0.60	126.00 ± 3.18	126.47 ± 5.19	14.21

CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

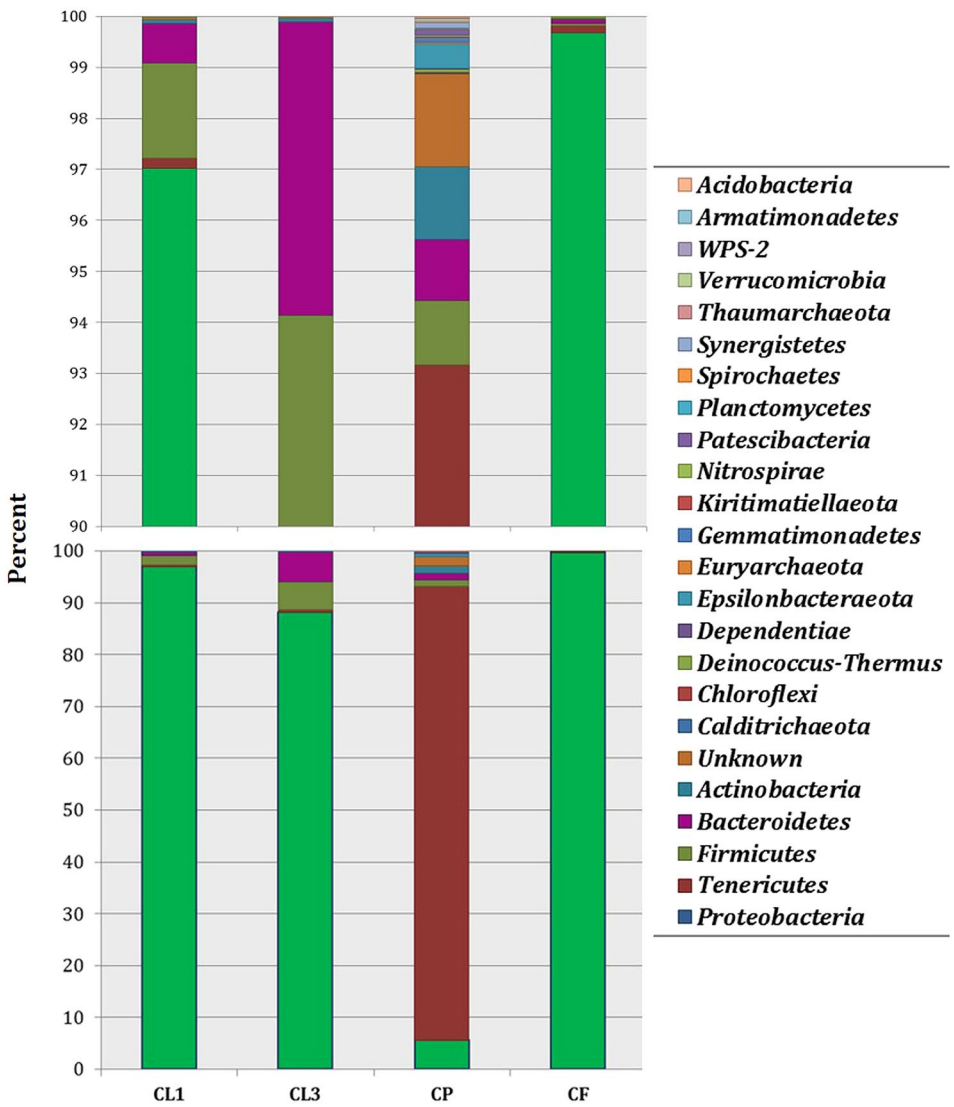

Fig. 2 Composition and relative abundance (%) of microbiota associated with *Zeugodacus cucurbitae* samples at different developmental stages at the phylum level. High-quality sequences obtained from different developmental stages were clustered in operational taxonomic units (OTUs) according to the open-reference method at a 97% of similarity

whereas Tenericutes was the dominant in the pupal stage (mean 87.53% of total) (Supplementary file 1). Out of four core phyla, class Gammaproteobacteria (87.85–98.42% in larva and adult) and Alphaproteobacteria in phyla Proteobacteria, Bacilli and Erysipelotrichia in phyla Firmicutes, class Actinobacteria in Actinobacteria phyla and class Mollicutes (87.53% in pupal stage) in phyla Tenericutes were most dominant (Supplementary file 1). SIMPER analysis revealed that average dissimilarities in bacterial community structures across developmental stages mainly from pupal stage, primarily arises due to share of Proteobacteria and Tenericutes phyla (Table 4).

The core bacterial orders were represented by Enterobacteriales, Betaproteobacteriales, Pseudomonadales, Mycoplasmatales and Bacillales among most dominant classes. The most abundant bacterial family was Enterobacteriaceae

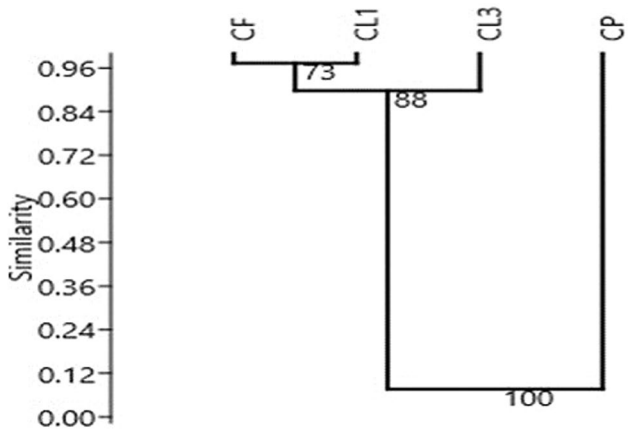

Fig. 3 UPGMA clustering showing relationship of microbiota associated with different developmental stages of *Zeugodacus cucurbitae* samples at the phylum level

(56.43–98.16%) across all developmental stages except in pupal stage (only 0.18%). In pupal stage, the most abundant family was Mycoplasmataceae (87.53%). Relative abundance of top picked 40 different families from different phylum showed that only a few number of bacterial OTUs displayed high relative abundance, as shown in the heat map of the OTUs (Fig. 4). *Providencia* and *Klebsiella* were the most dominant genus in larval stages (48.25–76.37%) and adult female stage (63.58%), respectively. Genus, *Candidatus*-Bacilloplasma lineage was dominant in pupal stage with 87.05 percent share (Table 5), whereas it was present in all the developmental stages (Supplementary file 1).

Functional Prediction of Genes

A total of 6909 KEGG Orthology (KO) database groups of metabolic functions were observed in terms of functional orthologs from predicted metagenomes of developmental

Table 4 Average dissimilarity in Bacterial community and Chi-square test values between developmental stages

Taxon	Average dissimilarity (%)					
	CL1-CL3	CL1-CP	CL1-CF	CL3-CP	CL3-CF	CP-CF
Proteobacteria	4.44	45.69	1.33	43.47	5.78	47.03
Bacteroidetes	2.49	43.66	0.91	41.25	2.84	43.70
Firmicutes	1.77	0.88	0.35	2.28	2.68	0.90
Tenericutes	0.20	0.68	0.04	2.07	0.24	0.71
Unknown	0.01	0.30	0.03	0.89	0.03	0.61
Deinococcus-Thermus	0.01	0.24	0.01	0.68	0.01	0.56
Armatimonadetes	0.00	0.21	0.01	0.24	0.01	0.24
Actinobacteria	0.00	0.06	0.00	0.06	0.00	0.06
Thaumarchaeota	0.00	0.05	0.00	0.05	0.00	0.05
Epsilonbacteraeota	0.00	0.04	0.00	0.04	0.00	0.04
Dependentiae	0.00	0.02	0.00	0.03	0.00	0.02
Nitrospirae	0.00	0.02	0.00	0.02	0.00	0.02
Patescibacteria	0.00	0.02	0.00	0.02	0.00	0.02
Planctomycetes	0.00	0.02	0.00	0.02	0.00	0.02
Kiritimatiellaeota	0.00	0.02	0.00	0.02	0.00	0.02
Gemmatimonadetes	0.00	0.01	0.00	0.01	0.00	0.01
Chloroflexi	0.00	0.01	0.00	0.01	0.00	0.01
Synergistetes	0.00	0.01	0.00	0.01	0.00	0.01
Euryarchaeota	0.00	0.01	0.00	0.01	0.00	0.01
Spirochaetes	0.00	0.01	0.00	0.01	0.00	0.01
Verrucomicrobia	0.00	0.01	0.00	0.01	0.00	0.01
WPS-2	0.00	0.01	0.00	0.01	0.00	0.01
Calditrichaeota	0.00	0.01	0.00	0.01	0.00	0.01
Acidobacteria	0.00	0.00	0.00	0.00	0.00	0.00
Overall	8.92	92.00	2.68	91.21	11.58	94.06
	Chi-square test (df = 24)					
X^2 value	69.83	951.08	22.06	857.67	89.78	857.90
P value	$P < 0.01$	$P < 0.001$	NS	$P < 0.001$	$P < 0.01$	$P < 0.001$

CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

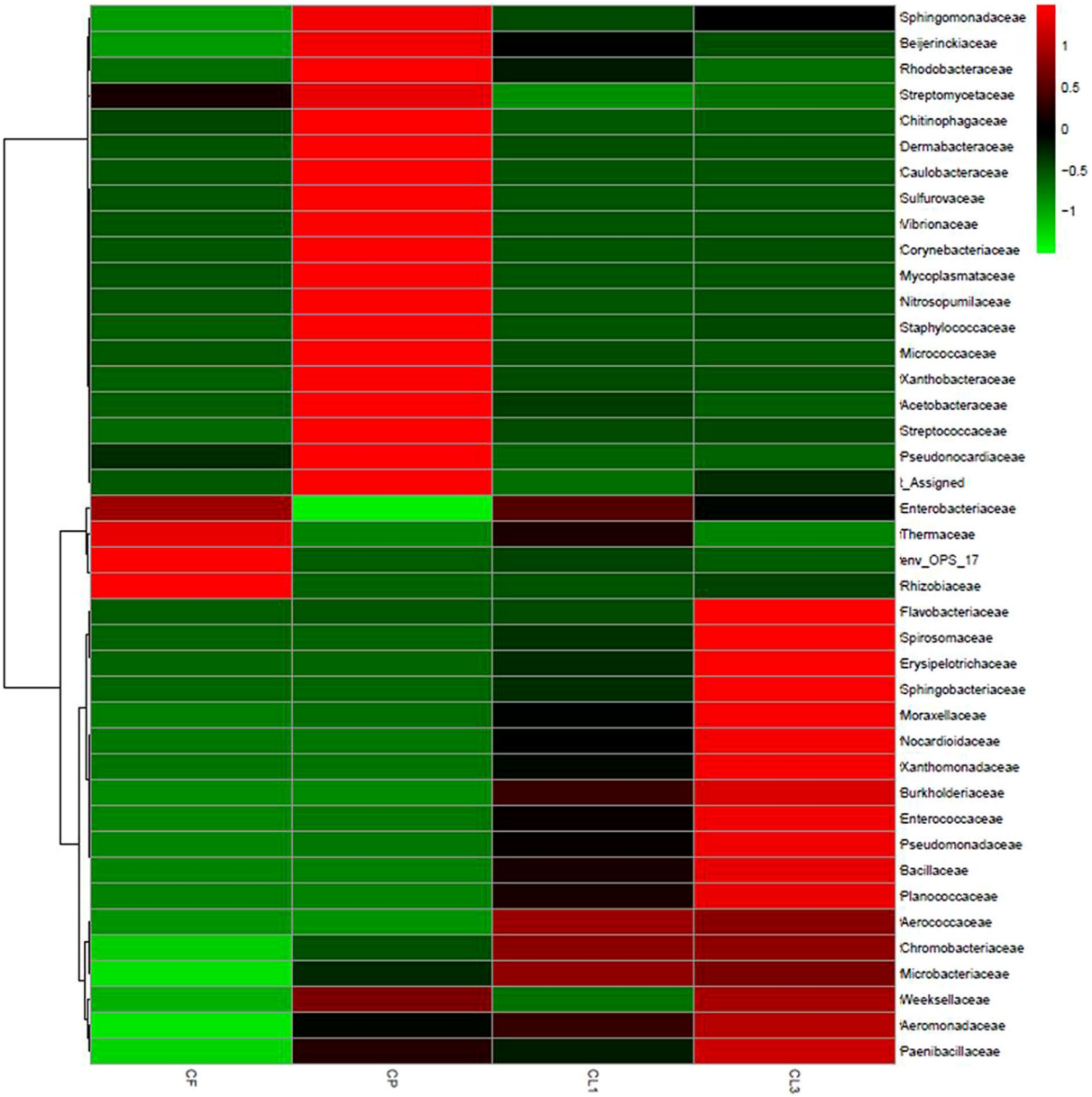

Fig. 4 Heat maps showing the relative abundances of 16S rRNA gene OTUs between different developmental stages, at family level. The colours indicate relative abundances ranging from green (lower abundances) to red (higher abundances) (indicated in scale of -1 to 1). CL1, CL3, CP and CF refer to 1st Instar larvae, 3rd Instar larvae, Pupa and Adult female stage of *Zeugodacus cucurbitae*

stages of *Z. cucurbitae*. The KO database analysis showed that bacterial community associated with developmental stages had rich in genes which may be involved in membrane transport, metabolism of carbohydrate and amino acid, energy metabolism, replication and repair processes as well as cellular processes and signalling (Fig. 5). Functional category pathways, i.e. membrane transport, signal transduction and transcription are predicted higher in larval and adult stages as compared to pupal stage. Pathways of energy metabolism, metabolism of cofactors, vitamins, nucleotide, replication and repair functional groups were higher individually in pupal stage (Fig. 5). Decreased activity of carbohydrate metabolism and enzymes families predicted from adults to larval stage, while vice versa in lipid metabolism.

Membrane transport system genes may be associated with the MFS (Major Facilitator Superfamily) transporters which are capable of transporting small solutes in response to ion gradients, ABC (ATP-Binding Cassette) transporters carry out ATP coupled transportation of small ions to macromolecules and phosphotransferase system (PTS) involved in the uptake of carbohydrates and convert them into their

Table 5 Top three genera along with family, class and phylum of microbiota associated with different developmental stages of *Z. cucurbitae*

Developmental Stages *Z. cucurbitae*	Genera	Family	Class	Phylum	Relative Abundance (in percent)
CL1(I Instar)	*Providencia*	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	76.37
	Comamonas	Burkholderiaceae	Gammaproteobacteria	Proteobacteria	13.47
	Klebsiella	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	2.08
CL3 (III Instar)	*Providencia*	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	48.25
	Comamonas	Burkholderiaceae	Gammaproteobacteria	Proteobacteria	24.04
	Acinetobacter	Moraxellaceae	Gammaproteobacteria	Proteobacteria	5.36
CP (Pupae)	*Candidatus*-Bacilloplasma	Mycoplasmataceae	Mollicutes	Tenericutes	87.05
	Photobacterium	Vibrionaceae	Gammaproteobacteria	Proteobacteria	1.17
	Shewanella	Shewanellaceae	Gammaproteobacteria	Proteobacteria	0.68
CF (Adult Female)	*Klebsiella*	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	63.58
		Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	15.59
	Citrobacter	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	7.35
	Pectobacterium	Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	6.45

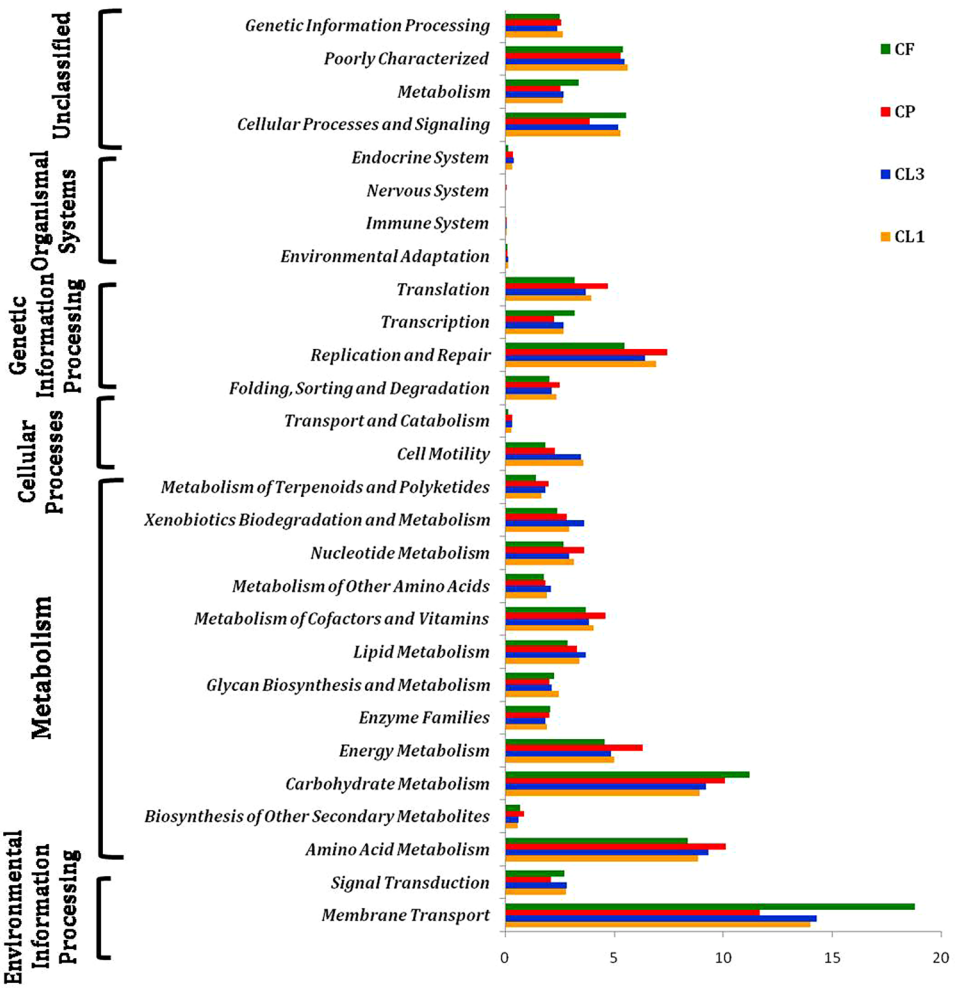

Fig. 5 Predicted Metabolic functions of bacterial communities associated with different developmental stages of *Zeugodacus cucurbitae*. All of the predicted KEGG metabolic pathways are shown at the second hierarchical level and grouped by major functional categories [50]

respective phosphoesters during transport. The predicted carbohydrate metabolism genes were abundant and greater number of OTUs were annotated for phosphoglycerate mutase and transketolase in adult compared to larvae and pupae, which involved in glycolysis and Pentose-phosphate pathway, respectively (supplementary file 3).

Discussion

The present study is first ever report of Illumina sequencing and annotation of bacterial community associated with different developmental stages of *Z. cucurbitae*. Earlier studies on the bacterial community associated with *Z. cucurbitae* were restricted to culture-dependent techniques or molecular approaches with low resolution [16, 17] or Illumina sequencing limited with adult flies [12, 18]. Illumina sequencing used in the present study reveals higher number of bacterial communities than those determined by culture-dependent techniques or other low resolution molecular approaches [7, 9, 11, 18]. Including the replications of test stages is always good for testing the differences within selected developmental stages [7, 9–13] but previous studies on different developmental stages of fruit flies based on single sample of stages showed high bacterial diversity through next generation sequencing [31–34]. We found 130 genera of different bacterial communities present in various developmental stages of *Z. cucurbitae*. The number of genera identified from different developmental stages of *Z. cucurbitae* is almost double from the study of Yong et al.[18] who used only males of *Z. cucurbitae* in their study and reported 67 genera of bacteria of which only 27 genera were common with the present study. However, only 40 genera of 130 genera detected from different developmental stages of *Z. cucurbitae* were found common with previous reports on microbiota of *Z. cucurbitae* by Hadapad et al. [16, 19], Yong et al. [18] and Asimakis et al. [35]. This suggests that *Z. cucurbitae* still has vast unexploited diversity of bacterial communities associated with different life stages. In present study, we were able to confirm the changes in the bacterial diversity during different development of *Z. cucurbitae*. Pupal stage was dominated by phylum Tenericutes, while phylum Proteobacteria was most predominant phylum in the larval and adult stages of *Z. cucurbitae*. Proteobacteria as the predominant phylum associated with developmental stages is consistent with the previous reports on fruit flies, i.e. *B. dorsalis*, *B. carambolae*, *B. minax*, *B. tryoni, B. neohumeralis, B. jarvasi* and *B. cacuminata* and adult male of *Z. cucurbitae* [9, 10, 18, 33, 36, 37]. In the present study, Tenericutes dominated in pupal stage, different from previous published studies on fruit flies but it is consistent with the report of Andongma et al. [31] where *B. dorsalis* bacterial diversity changes with developmental stages. The predominant phylum, Tenericutes association with pupal stage indicates that the pupal stage need some specific microbiota for different functions as also reported in earlier study on *B. dorsalis* [9]. Insect gut bacteria mostly dominated by phyla Proteobacteria and Firmicutes, followed by Bacteroidetes, Actinobacteria and Tenericutes in which difference in community composition may be due to host's location, food, developmental stage, physiology, and phylogeny [12].

The results revealed that bacterial genera *Providencia* and *Comamonas* of family Enterobacteriaceae was dominant in larval stages and *Klebsiella* of same family in adult female, while genus *Candidatus*-Bacilloplasma of family Mycoplasmataceae was dominant in pupal stage of *Z. cucurbitae*. Presence of Enterobacteriaceae bacteria in adult and larval stages may help in sugar metabolism and also plays very important role in courtship and reproduction of fruit flies [9, 38]. The variation in bacterial community across developmental stages may also be due to difference in the process of digestion, exposure to the type of diet and their habitat during these life stages [7, 37]. The presence of variety of digestive enzymes and association of metabolic capable symbiotic bacteria in insects make them suitable for wider environment adaptability and polyphagy [39]. The dominant bacterial genera present in the *Z. cucurbitae* were *Providencia* (larval stages), *Candidatus*-Bacilloplasma (pupal stage), *Comamonas* (larval stages), *Acinetobacter* (larval stages), *Bacillus* (larval stages), *Enterococcus* (larval stages and pupal), *Klebsiella* (adult female stage) *Citrobacter* (adult female stage), *Enterobacter* (adult female stage) and *Pectobacterium* (adult female stage). At genus level, *Providencia* was the most abundant genus in larval stages which gradually deceased with progression of *Z. cucurbitae* development stages. Genus, *Candidatus*-Bacilloplasma was the most dominant in pupal stage and it has very low abundance in other developmental stages (Table 5; Supplementary file 1). *Candidatus*-Bacilloplasma is a novel lineage of Mollicutes was reported to be associated with the hindgut wall of this terrestrial arthropod, *Porcellio scaber* [40]. Symbiotic association of Mollicutes in the intestinal tract of arthropods is a very common and found beneficial for the host [41]. However, "*Candidatus* Erwinia dacicola" a coevolved symbiotic bacteria has been reported from olive fruit fly, *Bactrocera oleae* [42]. The variation in the bacterial communities across developmental stages of *Z. cucurbitae* may occur due to their habitat during these life stages. In addition to the habitat, the transmission patterns of different bacteria species may affect its presence in the different life stages [31]. The difference in relative abundance of associated bacteria of *Z. cucurbitae* in present study compared to other [18] can be recognized to geographical isolations. The diversity and abundance of bacterial community within the species can also vary with pH, partial oxygen pressure and presence of physical barriers [3, 4]. From our results, only 15 OTUs were shared among all developmental stages of *Z. cucurbitae* (Fig. 1a) suggesting the

possibility of vertical transmission of these bacteria. Vertical transmission of bacteria in tephritids have been reported and maintained during the ontogeny of fruit flies [43]. Enterobacteriaceae family members have been reported to be vertically transmitted in Mediterranean fruit fly, *Ceratitis capitata* [44].

Fruit flies species usually feed upon nitrogen deficit fruits and vegetables subsequently cannot be able to synthesize some essential amino acids. Bacterial symbionts indirectly help in rotting of fruit fly infested fruits which increased the total amino acids for nutrition [45]. The association of symbiotic bacteria in fruit flies is necessary for their nutrition, defence, development and fitness in to environmental changes [14, 15, 46, 47]. Finally, the results of the present study based on functional prediction of bacterial community associated with different developmental stages of *Z. cucurbitae* suggest the involvement of bacterial community in various functions like metabolism of carbohydrate and amino acid and synthesis of protein as the fruit flies bred on low protein content host. The amino acid and carbohydrates metabolism could be important metabolic functions of bacterial community in insects feeding on low nitrogen content diets [48, 49].

In summary, this is the first report of bacterial diversity and abundance with their functional prediction associated during developmental stages of *Z. cucurbitae* using Illumina-based sequencing. The results of present study also significantly supplement the information to available literature and greater understanding of the bacterial diversity in *Z. cucurbitae* for development of novel pest management strategies.

Author Contributions JSC, NN, BD and BPB designed the study; NN and JSC carried out the experiments; NN, JSC and CSP analysed the data; NN, JSC, CSP, BD, AKS and BPB shared in scoping the study, data interpretation and writing the manuscript. All the authors have read and approved the final manuscript.

Data Availability Final sequence read achieves (SRAs) were deposited in the NCBI SRA database under the Bioproject accession ID PRJNA587221.

Compliance with Ethical Standards

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

Ethical Approval The study does not involve endangered or protected species and did not require any permission to conduct the experiment.

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