ORIGINAL ARTICLE

Developmental stage-associated microbiota profile of the peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae) and their functional prediction using 16S rRNA gene metabarcoding sequencing

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Abstract

The different developmental stage-associated microbiota of the peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae), was characterized using 16S rRNA gene (V3–V4 region) metabarcoding on the Illumina HiSeq platform. Taxonomically, at 97% similarity, there were total 16 bacterial phyla, comprising of 24 classes, 55 orders, 90 families and 134 genera. *Proteobacteria*, *Firmicutes, Actinobacteria* and *Bacteroidetes* were the most abundant phyla with *Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacteroidia* and *Bacilli* being the most abundant classes. The bacterial genus *Enterobacter* was dominant in the larval and adult stages and *Pseudomonas* in the pupal stage. A total of 2645 operational taxonomic units (OTUs) were identified, out of which 151 OTUs (core microbiota) were common among all the developmental stages of *B. zonata*. The genus *Enterobacter, Klebsiella* and *Pantoea* were dominant among the core microbiota. PICURSt analysis predicted that microbiota associated with *B. zonata* may be involved in membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair processes as well as in cellular processes and signalling. The microbiota that was shared by all the developmental stages of *B. zonata* in the present study could be targeted and the foundation for research on microbiota-based management of fruit flies.

Keywords *Bactrocera zonata* · Ontogeny · Microbiota · 16S rRNA gene · Management

Introduction

The dipteran flies of family Tephritidae are commonly known as fruit flies. The peach fruit fly, *Bactrocera zonata* (Saunders), is a serious insect pest of fruit crops in many parts of the world particularly in the South and South East Asia and in some parts of Africa with more than 50 plant species as host (Kapoor, 1993; Duyck et al. 2004; Choudhary et al. 2012; CABI/EPPO, 2013). Due to huge damage potential, *B. zonata* is considered as one of the most destructive insect pest species of peach, guava and mango in tropical and subtropical climatic conditions (Choudhary et al. 2012). Most of the studies related to this pest have been limited to its biology, distributions, population genetic structure, host plant association and management through pheromone-based mass trapping as well as with chemical insecticides (Duyck et al. 2004; Choudhary et al. 2012, 2018). However, very few studies have been carried out on the bacterial communities associated with *B. zonata,* limited to the culture-dependent techniques (Reddy et al. 2014; Naaz et al. 2016), whereas diversity and functional role of bacterial communities has been investigated in many insects groups including fruit flies (Fitt and O'Brien 1985; Lloyd et al. 1986; Prabhakar et al. 2009; Nakajima et al. 2005; Noman et al. 2019).

The bacterial communities in insects mostly live in the gut, hemocoel, mycetomes and within the cells. The bacteria plays a significant role in various metabolic functions of

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insects, i.e. nutrition, digestion, protection, detoxification of insecticides, extreme thermal tolerance and a source of cues and signals synthesis (semiochemicals) (Dillon and Dillon 2004; Russell and Moran 2006; Oliver et al. 2003; Engel and Moran 2013; Feldhaar, 2011; Douglas, 2015; Hammer and Bowers 2015; Ezenwa et al. 2012; Wingfield et al. 2016; Hosokawa et al. 2017). The insect–bacterial interaction is influenced by various factors like environmental conditions (Parmentier et al. 2016; Hongoh et al. 2006; Zhao et al. 2018; Malacrinò et al. 2018), diet and the host on which insects feed (Ferrari et al. 2007; Hosokawa et al. 2017; Wagner et al. 2015; Medina et al. 2011). Inter- and intraspecific variations have been reported in the bacterial communities of the insects' species and at different stages of insects' development (Aharon et al. 2013; Malacrinò et al. 2018).

Recent technology development and availability of cheaper DNA sequencing facilities have enabled the entire metabarcoding profiling and functional role of bacterial communities associated with different stages of insects including fruit flies of genus *Bactrocera* and *Ceratitis* (Wang et al. 2013; Wang et al. 2014; Zhao et al. 2018; Yong et al. 2017; Malacrinò et al. 2018; Aharon et al. 2013). However, the profiling of bacterial communities with recent molecular biology culture-independent techniques has not been investigated in the peach fruit fly, *Bactrocera zonata*.

Based on the current knowledge (Aharon et al. 2013; Andongma et al. 2015; Zhao et al. 2018; Malacrinò et al. 2018), we assumed that the gut microbiota in fruit flies changes with developmental stages (metamorphosis), and this leads to changes in molecular and phenotypic characters that provide the spatio-temporal adaptive advantages to the insect in any environment (Abdelfattah et al. 2017; Malacrinò et al. 2018). Therefore, in the present study, we examined the bacterial community harboured by *B. zonata*, during ontogeny using metagenomic techniques as well as predicted the functional metabolic activities of the associated microbial communities.

Materials and methods

Ethics statement

The study did not require any permits as it did not involve endangered or protected species.

Sampling for stage-associated microbiota of *B. zonata*

Fruit flies infested wood apple (*Aegle marmelos* L.) fruits were collected from Research farm of ICAR Research Complex for Eastern Region, Research Centre (ICAR-RCER, RC), Ranchi, India (23° 45′ N latitude, 85° 30′ E longitude,

elevation 620 m AMSL), in May 2018. Four different developmental stages, i.e. first instar larvae, third instar larvae, pupal stage and adult females, were collected. The first instar larvae were directly collected by cutting the infested fruits of wood apple with fruit fly. Different stages of *B. zonata* were reared on same host fruits in the laboratory conditions according to protocol described by Choudhary et al. (2020) and collected for further experiments. Larvae that emerged from the fruits and moved out to the sand for pupation were collected for the third instar larval stage. A fair number of third instar larvae were left in the soil to pupate in order to obtain pupal stage samples, and other pupae were left for the emergence of adult stage. 5-day-old adult females were collected to study the microbiota associated with the adult stage. All samples (first instar larvae, third instar larvae, pupae and adult females) were placed separately in microtubes and stored in − 80 °C until DNA extraction. Twenty specimens ($N = 5$ for each developmental stage) were used to compare the microbiota profile of different developmental stages of *B. zonata*.

DNA extraction, 16S rRNA gene amplification and sequencing

Each sample was separately surface sterilized with 0.1% mercuric chloride for 30 s followed by washing twice with double distilled H_2O for 60 s to remove the external contaminations. Then, total DNA was extracted from individual sample using the DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The quantity and quality of the extracted DNA were subsequently checked by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Each stage, DNA of five individuals of *B. zonata* were pooled together to make a single DNA sample for each developmental stage. The pooled DNA of each stage was further used for stage-associated microbiota analysis. The characterization of bacterial community was achieved by targeting the V3-V4 hypervariable regions of 16S rRNA gene with primers 341F (5′-TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGG GCTCGGAGATGTGTATAAGAGACAGGACTACH-VGGGTATCTAATCC-3′). Each developmental stage's library was prepared with a NEBNext Ultra II DNA Library Preparation Kit (New England BioLabs Inc.) according to the manufacturer's protocol using NEBNextQ5® Hot HiFi PCR Master Mix (NEBNext- New England BioLabs, and the NEBNext oligos kit (2×250 bp). Paired-end (PE, 2×250 nt) sequencing was performed with an Illumina HiSeq (HiSeq Rapid SBS Kits v2) at AgriGenome Labs, Cochin, Kerala; following the manufacturer's run protocols (Illumina, Inc., San Diego, CA, USA).

Sequencing data analysis and statistical analysis

The data from each developmental stage were processed and analysed using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) pipeline (Caporaso et al. 2010a). The total raw sequence reads were quality checked for different parameters, viz. base quality parameters, base composition distribution and GC distribution. Paired-end reads from HiSeq 2500 sequencing were quality trimmed and joined using FLASH program (Version 1.2.11, < https ://ccb.jhu.edu/software/FLASH/ >) (Magoč and Salzberg 2011). The consensus sequences were obtained after removing the low-quality read that did not meet the criteria for further analysis. As a part of pre-processing of sequence reads, chimeras were also removed with de novo (uchime_ denovo command) using the UCHIME algorithm implemented in the tool VSEARCH, version1.7.0 (Rognes et al. 2016). Pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using Uclust programme, version 1.2.22q1 (similarity cut-off = 0.97) (Edgar 2010) and assigned taxonomy using the Silva release 13.5 database as the reference126, with the taxonomy assignment tool PyNAST (Caporaso et al. 2010b). The downstream analyses of the core data were done using Biological Observation Matrix (BIOM) file. The sequences that did not have any alignment against taxonomic database and that were classified as mitochondria or chloroplast, as well as singletons, were filtered out of the data set. Bacterial diversity indices and Heatmap of relative abundance were generated using online Microbiome Analyst platform with the Euclidean distance method (Dhariwal et al. 2017). A Venn diagram to determine the unique and shared OTUs across the developmental stages of *B. zonata* was drawn using the VennDiagram package in R environment (https:// cran.r-project.org/bin/windows/base/). Similarity percentage (SIMPER) analysis was performed to calculate the average dissimilarities in structure of bacterial community between different developmental stages of *B. zonata*. The difference in structure of bacterial community was tested with χ^2 test in the PAST 3.25 software package (Hammer et al. 2001).

A phylogenetic tree was constructed with the four most abundant phyla of bacterial community of *B. zonata*. The longest reads of each bacterial family from four most abundant phyla were selected to compute the maximum likelihood tree with Tamura-Nei as the best fit model with 1000 bootstrap using MEGA 6.0 software (Tamura et al. 2013) and visualized using Interactive Tree Of Life (iTOL version 4.0) (Letunic and Bork 2019). The metabolic functions of the bacterial communities identified from the 16S rRNA sequences were predicted by annotating pathways of OTUs against the KEGG database using PICRUSt v. 1.1.4 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved State) software (Langille et al. 2013).

Results

Description of 16S rRNA gene sequencing reads

Illumina HiSeq platform was used to sequence the 16S rRNA gene of microbiota associated with different developmental stages of *B. zonata*. A total of 8,73,093 raw sequence reads of 16S rRNA gene were generated from different developmental stages of *B. zonata*. Finally, a data set for each stages of *B. zonata* was prepared after the removal of low quality and chimera from raw sequences (Table 1). Overall, 2645 OUTs were identified from all stages of *B. zonata* (Table 1). Among them, the lowest number of OTUs (Operational Taxonomic Units) was observed from the pupal stage (BP; 727) and highest number from the first instar larval stage (BL1; 1442) which indicates the complexity of microbial population across the developmental stages (Table 1). The Venn diagram showed that 97% OTUs were shared among different developmental stages of *B. zonata* (Fig. 1a). A total of 151 OTUs (core microbial community) were common across each stage. Percentage-wise distribution of core microbial community (151 OTUs) reveals that *Proteobacteria* (> 98%) were the most dominant phylum in each stage, whereas *Firmicutes* and *Tenericutes* contributed less

Table 1 Summary of the 16 s rRNA sequencing data

ID[b]	No. of bacterial reads	Total consensus sequences	Pre-processed consensus sequences	No. of observed OTUs[a]	GC content (%)	Avg. length (nt)
BL1	250,632	143,077	118,103	1442	54.63	450
BL3	203,871	121,364	111,049	1098	50.66	450
BP	216,637	110,562	66,528	727	50.58	450
BF	201,953	121,261	114,815	1221	50.57	450
Total				2645		

[a]OTUs (operational taxonomic units) at the 97% sequence similarity cut-off

[b]BL1, BL3, BP and BF refer to first instar larvae, third instar larvae, pupa and adult female of *Bactrocera zonata*

Fig. 1 OTUs (Operational Taxonomic Units) analysis between different developmental stages of *B. zonata* at 97% similarity. **a** Venn diagram showing unique and shared OTUs, of which 151 OTUs shared between all developmental stages. **b** Percentage distribution of common shared OTUs (151 OTU) between all developmental stages at phylum level. BL1, BL3, BP and BF refers to first instar larvae, third instar larvae, pupa and adult female of *Bactrocera zonata*
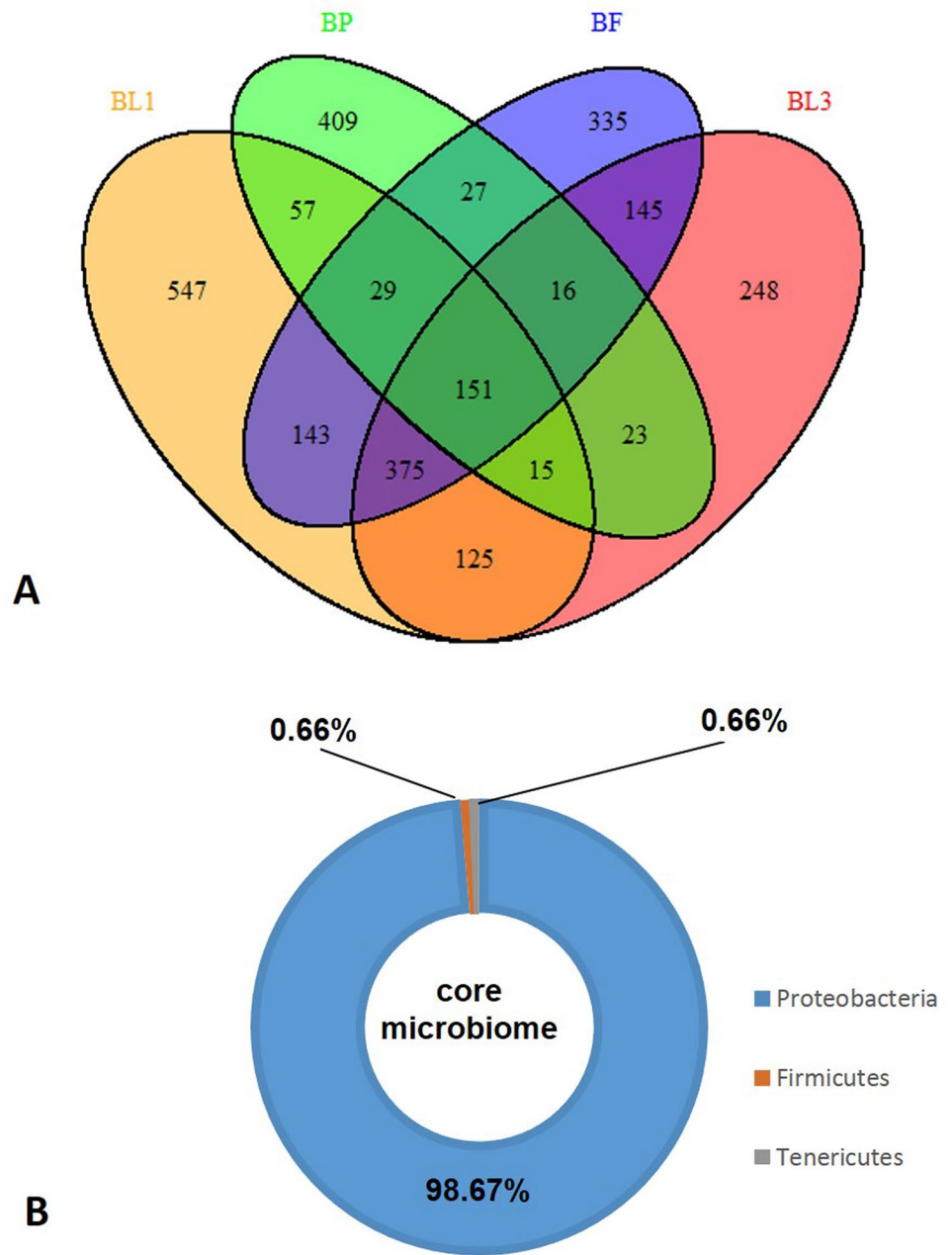
than 1% (Fig. 1b). Shannon and Simpson diversity index analysis indicated the highest species richness of bacterial communities in pupal stage of *B. zonata* (Table 2). The rarefaction curves for the BP (Supplementary Fig. 1) did not attain full plateau, suggesting that bacterial richness in pupal stage of *B. zonata* was not yet determined completely. Additional sequence sampling is still required to capture the microbiota diversity of *B. zonata* pupal stage. The taxonomic analyses of all developmental stages are available as supplementary material (Supplementary Table 1) and with clear understanding through sunburst charts of each stage (Supplementary Fig. 2; html links).

Table 2 Diversity of bacterial community in different developmental stages of *Bactrocera zonata*

ID[a]	Shannon index	Simpson index	Chao 1	Ace	Fischer
BL1	1.89	0.55	716.66	756.11	104.18
BL3	1.19	0.32	757.42	705.14	97.46
BP	2.44	0.75	315.63	310.75	44.95
BF	1.29	0.32	726.24	720.56	100.28

[a]BL1, BL3, BP and BF refer to first instar larvae, third instar larvae, pupa and adult female of *Bactrocera zonata*

The taxonomic composition of microbiota associated with different developmental stages

Microbiota associated with *B. zonata* in the present study were categorized into 16 bacterial phyla including an unassigned bacterial phylum, comprising of 24 classes, 55 orders, 90 families and 134 genera. Among bacterial phyla, *Proteobacteria*, *Firmicutes, Actinobacteria* and *Bacteroidetes* were the most abundant across the developmental stages with varying percentages (Table 3 and Fig. 2). *Proteobacteria* were consistently predominant phylum in all developmental stages (Fig. 2). *Proteobacteria* dominated with an average of 93.7% (91.72% in BL1 to 95.67% in BF) is followed by *Firmicutes* with an average 2.6% (0.22% in BP to 6.52% in BL3), *Actinobacteria*, 1.3% (0.02% in BL3 to 3.52% in BP), and *Bacteroidetes*, 0.43% (0% in BL3 to 1.41% in BP) of the total reads (Fig. 2 and Supplementary Table 1). However, other 12 phyla, viz. *Acidobacteria, Tenericutes, Verrucomicrobia, Epsilonbacteraeota, Deinococcus-Thermus, Gemmatimonadetes, Epsilonbacteraeota, Nitrospirae, Patescibacteria, Chloroflexi, Spirochaetes, Euryarchaeota* and other unclassified phylum, were found at very low relative abundance. The exception was *Tenericutes* in first instar larvae (BL1) which contributed 6.11% of the total reads in this stage. SIMPER analysis revealed that average dissimilarities in the bacterial community structure across developmental stages were primarily due to the sharing of phyla *Proteobacteria* and *Actinobacteria* between stages (Table 3).

Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacteroidia and *Bacilli* were the predominant classes of bacterial communities found in the present study. These comprised more than 93% of total reads of all the developmental stages. The most abundant class was *Gammaproteobacteria,* accounting more than 90% individually in all developmental stages except in the pupal stage (BP) (74.93%) followed by class *Alphaproteobacteria* (19.09%) (Supplementary Table 1). Across all the developmental stages except pupal stage, the most abundant family was *Enterobacteriaceae* ranged from 90.10% in first instar (BL1) to 95.35% in adult fly (BF). The pupal stage was only accounted for 22.71% in *Enterobacteriaceae* because 53.15% of *Proteobacteria* reads were not classified up to family level. Therefore, the most abundant family in the pupal stage was *Pseudomonadaceae* (28.62%). When OTUs assigned to the genus level, genus *Enterobacter* was the most dominant genus found across the developmental stages of *B. zonata*, which comprised of 64.69%, 83.48% and 82.63% in BL1, BL3 and BF stage, respectively. The pupal stage harboured *Pseudomonas* (28.62%) as the most abundant genus followed by *Enterobacter* (15.47%). However, the first instar larval stage consisted of *Candidatus-Bacilloplasma* lineage which comprised of 5.81%. (Supplementary Table 1).

Table 3 Average dissimilarity in bacterial community and Chi-square test values between developmental stages

Taxon	Average dissimilarity (%)					
	BL1-BL3	BL1-BP	BL1-BF	BL3-BP	BL3-BF	BP-BF[a]
Proteobacteria	2.92	4.47	2.31	7.39	0.61	6.78
Actinobacteria	0.97	2.33	0.45	3.30	0.52	2.78
Tenericutes	1.09	0.84	0.89	0.25	0.20	0.06
Firmicutes	0.10	0.27	0.07	0.17	0.17	0.34
Bacteroidetes	0.49	1.10	0.40	1.58	0.08	1.50
Euryarchaeota	0.10	0.10	0.10	0.00	0.00	0.00
Acidobacteria	0.10	0.03	0.10	0.14	0.00	0.14
Nitrospirae	0.03	0.03	0.03	0.00	0.00	0.00
Gemmatimonadetes	0.03	0.10	0.03	0.14	0.00	0.14
Chloroflexi	0.03	0.24	0.03	0.28	0.00	0.28
Patescibacteria	0.03	0.03	0.03	0.00	0.00	0.00
Spirochaetes	0.03	0.03	0.03	0.00	0.00	0.00
Deinococcus-Thermus	0.02	0.07	0.03	0.05	0.00	0.04
Epsilonbacteraeota	0.01	0.03	0.03	0.02	0.05	0.07
Verrucomicrobia	0.00	0.34	0.00	0.34	0.00	0.34
Unknown	0.08	1.13	0.05	1.22	0.03	1.18
overall	6.07	11.17	4.62	14.87	1.66	13.67
df =	11	14	10	12	9	12
chi square test	10.53	3.29	14.70	6.70	25.94*	6.70
	$p < 0.5$	NS	$p < 0.10$	NS	$p < 0.01$	NS

[a]BL1, BL3, BP and BF refer to first instar larvae, third instar larvae, pupa and adult female of *Bactrocera zonata*

Fig. 2 UPGMA clustering of *B. zonata* samples at different developmental stages according to community composition and structure with relative abundance of bacterial community 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. BL1, BL3, BP and BF refer to first instar larvae, third instar larvae, Pupa and Adult female of *Bactrocera zonata*
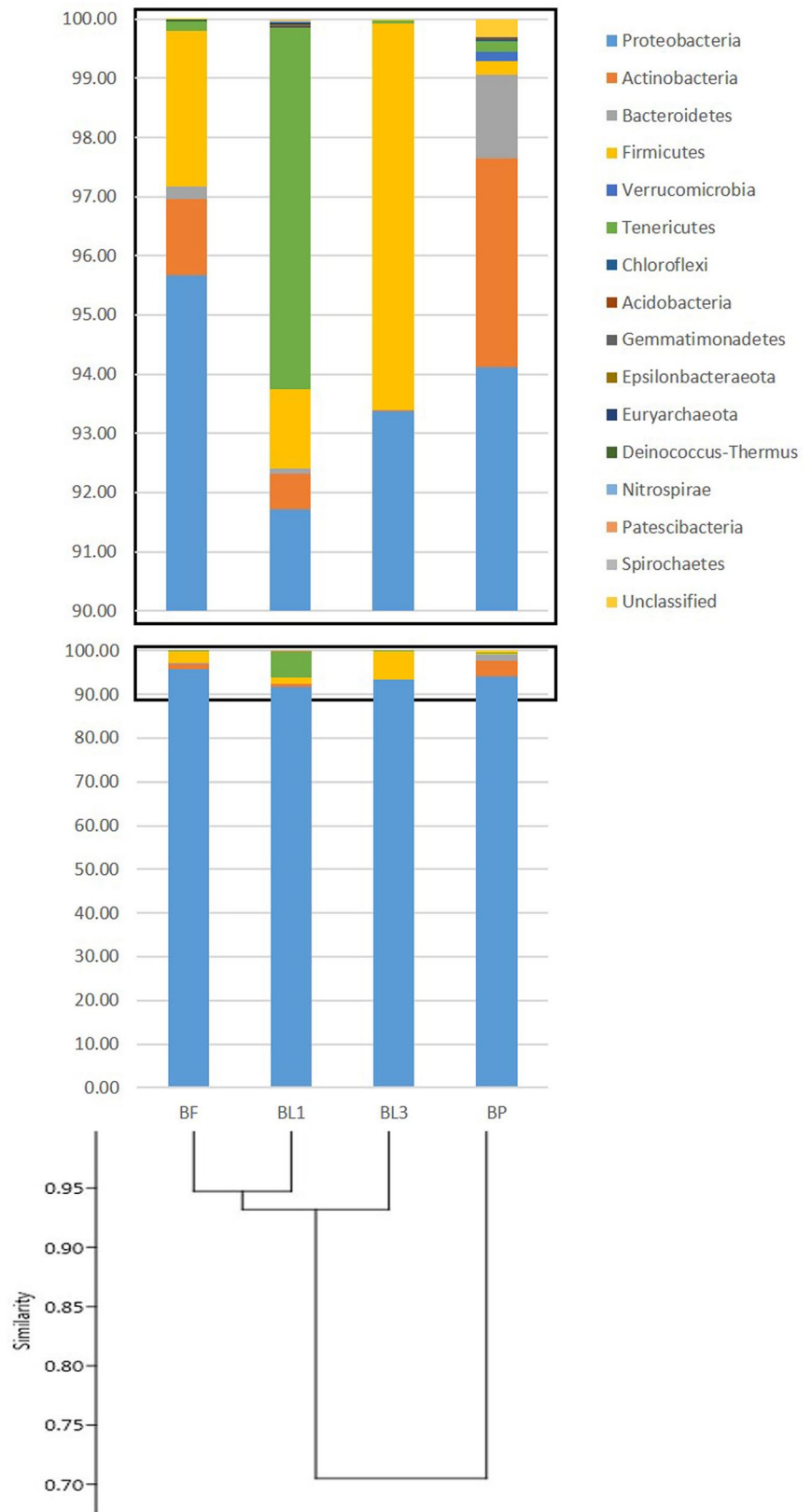
The different developmental stages of *B. zonata* were tested, representing top picked 37 different families from different phylum. Only a few bacterial OTUs displayed high relative abundance, as shown in the heat map of the OTUs (Fig. 3). UPGMA clustering of OTUs across developmental stages of *B. zonata* revealed that changes in microbiota composition were not associated with the development of successive stages (Fig. 2). Microbiota profile of third instar larvae and adult were the most similar, whereas microbiota associated with pupae was least shared with those associated with other developmental stages. Phylogenetically, bacterial families of phylum *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were placed in the respective single clade and *Proteobacteria* were further grouped into classes *Alphaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* (Fig. 4).

Functional prediction of microbiota across developmental stages

The functional potentials of microbiota associated with developmental stages of *B. zonata* were predicted using PICRUSt analysis. A total of 6538 KEGG Orthology (KO) groups were observed in the predicted metagenomes involving in membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair processes and cellular processes and signalling (Fig. 5). The highest number of functional potential of carbohydrate metabolism, replication

Fig. 3 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 a scale of − 1 to 1)
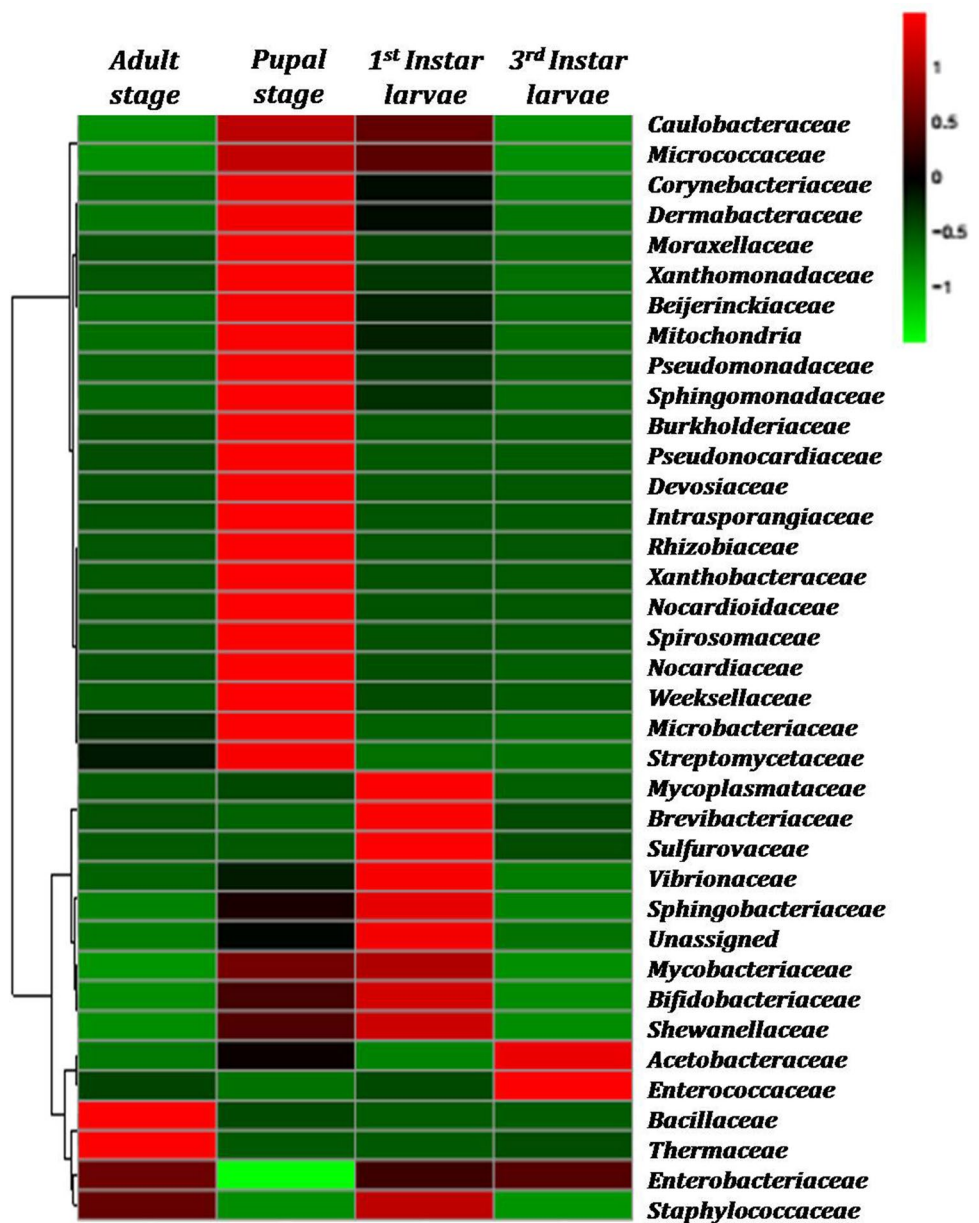
Fig. 4 Maximum likelihood tree constructed for phylogenetic analysis of most dominant taxa of bacterial community (on the basis of > 1% abundance of phylum in at least two developmental stages and present across all the developmental stage using 16S rRNA gene sequences) associated with the developmental stages of *B. zonata*. Bootstrap values were obtained from a search with 1000 replicates
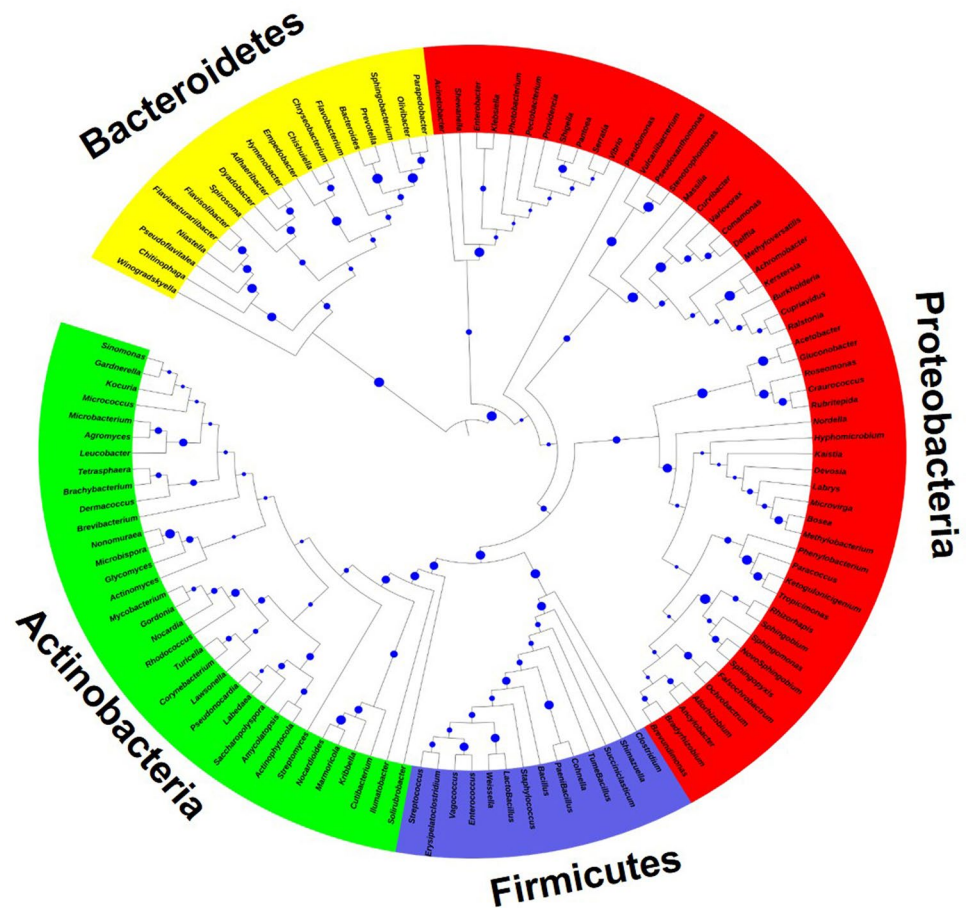
and repair processes, cellular processes and signalling and signal transduction of bacterial communities were predicted in the pupal stage compared to other stages. Based on the OTU abundance, some predicted pathways are likely to be more abundant in adult stage compared with the pupal stage (e.g. carbohydrate metabolism, replication and repair processes, energy metabolism as well as cell motility).

Genes involved in the membrane transport system are assumed to be associated with the MFS (Major Facilitator Superfamily) transporters. MFS are capable of transporting small solutes in response to ion gradients, ATP-Binding Cassette (ABC) transporters transport a variety of substrates ranging from small ions to macromolecules coupled with the ATP hydrolysis. PTS (phosphoenolpyruvate-dependent phosphotransferase) system can also be involved in the uptake of carbohydrates and convert them into their respective phosphoesters during transportation (Supplementary Table 2). During ontogeny, the abundant OUT-predicted genes were present for the enzymes transketolase and phosphoglycerate mutase that were involved in two different carbohydrate metabolism pathways, viz. pentose-phosphate pathway and glycolysis, respectively. A large number of OTUs were annotated for the "antibiotic

transport system ATP-binding proteins" and the "antibiotic transport system permease proteins" in all developmental stages. The high numbers of OTUs were also recorded with proteases that were involved in the protein metabolism pathways (Supplementary Table 2).

Discussion

This is the first ever report using Illumina HiSeq sequencing that revealed the microbiota associated with *B. zonata* during its ontogeny. Previously, only the gut microbiota of *B. zonata* had been studied with culture-based and conventional molecular methods (Naaz et al. 2016; Reddy et al. 2014). These methods allow the identification of less than 1% of the insect's associated microbiota (Hill et al. 2000). Metabarcoding approaches on the Illumina HiSeq platform have proven better to resolve the complexity of the associated microbial community during the ontogeny in fruit flies (Yong et al. 2017; Zhao et al. 2018; Aharon et al. 2013; Malacrinò et al. 2018).
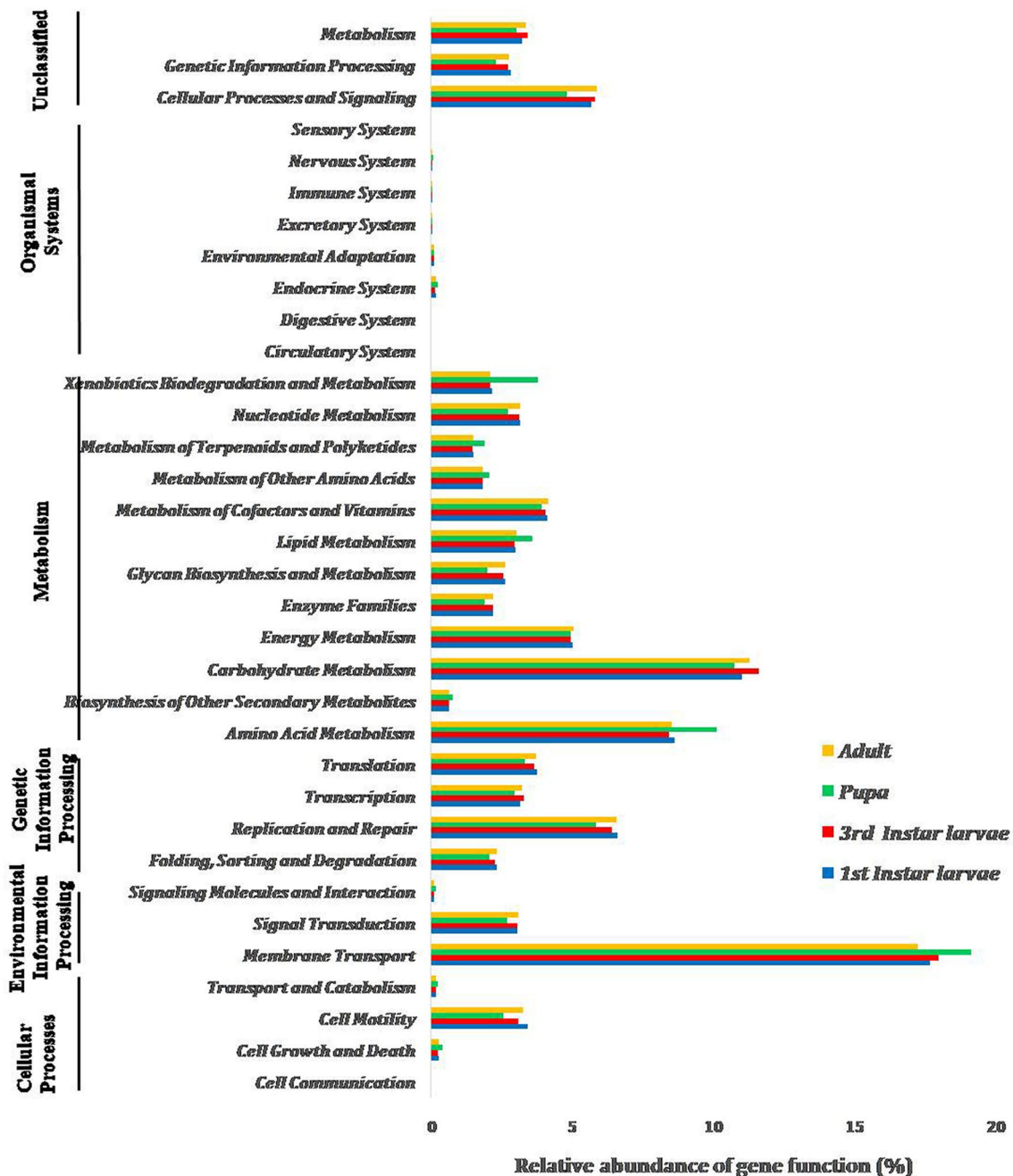
Fig. 5 Predicted metabolic functions of bacterial communities associated with the different developmental stages of *B. zonata*. All of the predicted KEGG metabolic pathways are shown at the second hierarchical level and grouped by major functional categories

Diversity and predicted functions of microbiota associated with *B. zonata*

The present study showed that *Proteobacteria* was dominant phylum associated across the developmental stages of *B. zonata*. This is consistent with the previous reports on microbiota associated with *Bactrocera* species, i.e. *B. dorsalis*, *B. carambolae*, *B. minax*, *B. tryoni*, *B. neohumeralis*, *B. jarvasi* and *B. cacuminata* (Wang et al. 2014; Yong et al.

2017; Morrow et al. 2015; Deutscher et al. 2018; Zhao et al. 2018; Augustinos et al. 2019). In contrast to this, *Firmicutes* was the dominant phylum in the adult stage of *B. dorsalis* (Andongma et al. 2015). In this study, the phylum *Actinobacteria* was the most predominant bacterial community in the pupal stage of *B. zonata* in comparison with immature (larvae) and adult stages. Recently, association of phylum *Actinobacteria* with pupal stage of *B. dorsalis* has been reported with their possible specific role in the pupal stage

of the fruit flies (Zhao et al. 2018), as both the *Bactrocera* species are very similar in their geographical distribution, climatic requirements, biology as well as their host plant association.

The host polyphagy and adaptation to a wide variety of environment by the insects is made possible by the presence of a variety of digestive enzymes and the association with metabolically capable symbiotic bacteria (Berasategui et al. 2016). Our result showed that members of *Enterobacteriaceae* were the most dominant family during larval and adult stage, whereas members of *Pseudomonadaceae* were the predominant in the pupal stage of *B. zonata.* The dominance of the members of *Enterobacteriaceae* during larval and adult stage of *B. dorsalis* may have their role in sugar metabolism (Zhao et al. 2018). The results suggest that the process of digestion may differ with the developmental stage, resulting in changes in the microbiota composition. Similarly, host diet is known to influence gut microbiota composition in fruit flies (Malacrinò et al. 2018). The change in gut bacterial diversity was observed when adults of *B. dorsalis* were fed with a full diet versus a sugar diet (Wang et al. 2011). The family *Enterobacteriaceae,* known to be associated with most of the fruit fly species, also play a vital role in courtship and reproduction (Ben Ami et al. 2010).

Fruit flies mostly feed on low-nitrogen containing fruits and vegetables and cannot be able to synthesize some of the essential amino acids. *Enterobacteriaceae* (diazotrophic bacteria) are known to play a role in atmospheric nitrogen fixation (Behar et al. 2005) and may also contribute to host fitness by limiting the proliferation of pathogenic bacteria found in the medfly, *Ceratitis capitata* (Behar et al. 2008). In the present study, members of *Enterobacteriaceae* were dominant ($> 90\%$) among bacterial communities across all the developmental stages of *B. zonata* except pupa ($< 23\%$). This suggests that *Enterobacteriaceae* may play a valuable role for the development of host fly, *B. zonata*. In the pupal stage, *Pseudomonas* was dominant followed by *Enterobacter* and *Achromobacter* ($> 10\%$ relative abundance) which is in line with the report on *B. carambolae* (Yong et al. 2017). *Pseudomonas* spp. is also reported as an entomopathogen in diamondback moth (Indiragandhi et al. 2007), and it can synthesize a toxic molecule with potential antiparasitic activity in mosquitoes (Azambuja et al. 2005) and low fitness in *Ceratitis capitata* (Behar et al. 2008). We report the association of genus, *Candidatus*-Bacilloplasma for the first time with *B. zonata* and found abundantly in the first instar (5.81%). *Candidatus*-Bacilloplasma is a lineage of *Tenericutes* which was first time described in *Porcellio scaber* (Kostanjsek et al. 2007). The presence of coevolved symbiotic bacteria has been reported in other fruit flies such as *Bactrocera oleae* which harbours "*Candidatus* Erwinia dacicola" (Capuzzo et al. 2005; Estes et al. 2012). So, the genus *Candidatus* may have specific role in the development of *B.*

zonata as symbiotic bacteria. The variations in the bacterial communities across developmental stages of *B. zonata* may reflect changes in the habitat during its life stages.

In the present study, 151 OTUs were common among all the developmental stages of *B. zonata* and suggest the possibility of vertical transmission of these bacterial communities (Fig. 1A). Vertical transition is common for some of the bacterial communities in tephritid flies (Andongma et al. 2015; Lauzon et al. 2009). The core microbiota of phylum *Proteobacteria* was comprised of predominant *Enterobacter* followed by *Klebsiella* and *Pantoea.* Some other *Proteobacteria* were also present in *B. zonata,* viz. *Pectobacterium, Providencia, Serratia* and *Shigella*. These bacterial communities have also been found in the gut of other tephritids (Wang et al. 2011; Liu et al. 2016, 2018; Yong et al. 2017; Morrow et al. 2015; Deutscher et al. 2018; Zhao et al. 2018; Augustinos et al. 2019). Our data are in support of previous studies, at least regarding the "key" players, mainly few genera belonging to *Gammaproteobacteria*. The genus *Stenotrophomonas* was found at low levels in the pupa ($\leq 0.5\%$) and in the first instar larvae ($< 0.01\%$) which was earlier recorded in the gut of *B. zonata* adult flies (Reddy et al. 2014) and also in the adult and larval stage of *B. dorsalis* (Thaochan et al. 2013; Yong et al. 2017). The diversity and abundance of the bacterial community in insect gut may be influenced by metamorphosis, pH, from acidic to extremely alkaline conditions in the gut compartments of various insects as well as the partial oxygen pressure from the outside environment (Moll et al. 2001; Dillon and Dillon 2004; Engel and Moran 2013).

The present study also provides predictions of the metabolic functions of the microbiota associated with developmental stages of *B. zonata*. PICRUSt analysis predicted the high abundance of genes involved in membrane transport (especially those connected with ABC transporters) (Fig. 5) which might be related to antibiotic resistance. This is because of ATP-binding cassette (ABC)-type multidrug transporters use a free energy of ATP hydrolysis to pump drugs out of the cell (Putman et al. 2000). The amino acid metabolism could be a vital metabolic function of microbiota for insects feeding on diets with low nitrogen content (Ben-Yosef et al. 2014). However, carbohydrates metabolism, especially pectin hydrolysis, could be an important in larval stage, when fruits have low protein content (Prabhakar et al. 2009). The predicted glutathione S-transferase gene with high abundance might have the ability to detoxify insecticides and xenobiotics (Hu et al. 2014). However, these in silico predicted functions need to be validated in vitro in future research.

The association of symbiotic bacteria in fruit flies is very important for their nutrition, defence, development and fitness to environmental changes. In the present study, information could be used to develop a target

symbioticide and manipulation of particular bacterial community harboured during ontogenesis process for the development of Integrated Pest Management programmes. Such pest management programmes have been explored earlier by researchers, i.e. *Wolbachia*-induced cytoplasmic incompatibility in the medfly (Zabalou et al. 2004), use of symbioticide for fruit fly management (Belcari and Bobbio 1999; Sood and Prabhakar 2009), attracting odours (Naaz et al. 2016), enhancing the success of sterile insect technique (Niyazi et al. 2004), declining the pesticide resistance (Cheng et al. 2017), mass rearing of parasitoids (Leroy et al. 2011) and so on (Prabhakar et al. 2008; Noman et al. 2019). In addition, the symbionts of insect are being exploited as promising sources for novel bioactive metabolites (Dettner 2011). Chemical defence mechanisms provided by microbial symbionts to their host against pathogens, parasites, parasitoids and predators have been studied in several insects, including planthoppers (Fredenhagen et al. 1987), beetles (Kellner 2002), psyllids (Nakabachi et al. 2013) and solitary wasps (Kaltenpoth and Engl, 2014). *Actinobacteria* are known as important insect's symbiont for providing defence for their host by producing antimicrobial activity compounds (Kaltenpoth 2009). The moderate abundance of *Actinobacteria* in the pupal stage of *B. zonata* as compared to other developmental stages supports the idea that *Actinobacteria* plays a defensive role in the pupal stage of *B. zonata*. This is consistent with the previous observation that *B. dorsalis* has abundant *Actinobacteria* in the pupae. The presence of *Dermabacteraceae* and *Streptomycetaceae* in pupae, in our study, may indicate a defensive function. Members of genus *Streptomyces* are well known as the main source of antibiotics with diverse biological activities (Arasu et al. 2008). The genus *Brachybacterium* of the family *Dermabacteraceae* were also identified and known to express strong antimicrobial activity (Liu et al. 2011). Therefore, pupae of *B. zonata* could be utilized as a source of bacteria with antimicrobial activity.

In conclusion, this study is the first report of the microbiota diversity and abundance associated during ontogenesis of *B. zonata* using next-generation sequencing. Distinct microbial flora association with specific developmental stages of *B. zonata* might suggest a specific role of microbial community to fulfil the developmental needs of particular stages in this insect. The results of the present study also supplement information to the available literature and provide a better understanding of the microbiota associated with *B. zonata* and other tephritids. Future research is needed to validate the in silico predicted functions of each taxa of microbiota in the in vitro conditions, identifying their capability as source of novel bioactive compounds as a bio-control agent, and the possibilities for targeted manipulation in future management programmes of fruit flies are an exciting research challenge.

Compliance with ethical standards

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