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BIODIVERSITY OF THE GUT MICROBIOTA OF *Epilachna vigintioctopunctata* USING ILLUMINA MiSEQ PLATFORM

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ABSTRACT

The insect gut is a complex system colonized by a consortium of microbes which share a symbiotic relation with the host and also contribute to its growth, development, immunity and defense against its enemies, reproduction and speciation, thereby play a crucial role in its survival. Hence understanding the role of these bacteria is a crucial step for using them in microbial control of pests. In present study, a 16S microbiome profiling of gut of *Epilachna vigintioctopunctata* was carried out. The study revealed 553 operational taxonomic units (OTUs). The majority of bacterial OTUs belonged to the phylum Proteobacteria, followed by Bacteroidetes, Firmicutes and Actinobacteria. The majority of bacterial OTUs belonged to the family Halomonadaceae which was the most abundant taxa in larval gut. The study provides a baseline information on gut inhabiting microbes of *E. vigintiocto-punctata* up to the genus level for most phyla.

Keywords: *Epilachna vigintioctopunctata*, gut bacteria, Halomonadaceae, Illumina MiSeq, Proteobacteria, 16s rRNA gene sequencing

INTRODUCTION

Insects have varied species-diversity and body mass in all the ecological habitats and are world's most diverse, abundant and successful group of animals (Nagarajan *et al.,* 2022). They interact with an array of microbes, especially those living in gut, which help them in growth and survival, as well as play a vital role in the biology and behaviour of the hosts (Tokuda *et al.,* 2018; Xia *et al.,* 2018); thereby depicting beneficial effects on host (Geng *et al.,* 2022). The digestive tract of insects is divided into three basic regions - foregut, midgut, and hindgut. The fore- and hind-guts are formed from the embryonic epithelium and have a chitinous exoskeleton with integuments made of glycoproteins which serve as a protective shield against pathogens (Siddiqui *et al.,* 2022). In majority of insects, midgut is the main location of absorption and digestion which is covered by a peritrophic matrix. This matrix is made up of chitin, proteins and proteoglycans, that separates the food content from the gut epithelium (Hegedus *et al.,* 2019) as well as protects the epithelium against mechanical damage, pathogens and toxins (Erlandson *et al*., 2019; Liu *et al*., 2019).

Understanding the role of microbes colonizing the insect gut is an important step towards the process of using bacteria in microbial control of insect pests. Insect guts are inhabited by different microbial species which are either benign or beneficial to their hosts. However, very little is known about how the microbial gut communities vary among different host species. In this study, a 16S

metagenomic sequencing of the gut of Hadda beetle (*Epilachna vigintioctopunctata* Fab.), commonly called as the 28-spotted ladybird, was carried out. The beetle belongs to the order Coleoptera of family Coccinillidae. It is one of the most important agricultural crop-pests causing a considerable damage to many commercial crops *viz*., *Solonum melongena, S. lycopersicum, S. tuberosum, Nicotiana tabacum, Cucumis melo, C. sativus* and cucurbits) (Rath, 2005; Manikandan *et al*., 2017) and some medicinal plants like *Datura innoxia*, *D. stromonium*, *Solonum nigrum*, *Physalis minima, Withania sominfera* and *Amaranthus caudatus* (Tara *et al*., 2017). Both grubs and adults of *E. vigintioctopunctata* feed on epidermal tissue of leaves by scrapping on leaf surfaces resulting in drying and eventually shedding of leaves. This, in turn, leads to retarded plant growth and reduction of fruit yield upto 60% (Mall *et al*., 1992; Bumpy *et al*., 2023). Microbial communities by large can fairly be understood by next-generation sequencing as it is one of the most useful and high-throughput sequencing technology associated with metagenomic and molecular biology in the characterization of gut microbiome of insects (Rajilic-Stojanovic and de Vos, 2014). Sogin *et al.* (2006) suggested that the culture-independent molecular approaches using 16S rRNA gene is most effective method to characterise gut microbiome of insects. The sequencing of 16S rRNA gene is a gold standard for the identification and taxonomic studies. The present study was aimed to describe the metagenomics of bacterial communities associated with the gut of *Epilachna vigintioctopunctata;* and gut microbiota were analysed in hypervariable regions of 16s rRNA gene sequences which may express major sequence diversity among different microorganisms. The sequences were clustered against a reference sequence collection and each clusters, known as Operational Taxonomic Unit (OTU) represented a taxonomic unit of microbial species or genus based on the sequence similarity threshold.

MATERIALS AND METHODS

The larvae of *E. vigintioctopunctata* were collected from Shettihalli (Bangalore rural, Karnataka) (13.7955 °N, 76.1711 °E) in the month of July 2022. Fourth instar larvae of *E. vigintioctopunctata* were used for the isolation and dissection. The larvae were starved overnight, so that gut remains clear of any food particle. The insects were immobilised by chloroform, surface sterilised in 0.1% sodium hypochlorite and 70% aqueous ethanol for 5 sec. to remove the adhering contaminants especially the external microflora (Gebbardi *et al.*, 2001). The insect larvae were then mounted on wax plates with water. The head capsule was properly fixed with help of a sharp pin and similarly the last segment also pinned. Care was taken not to damage the digestive system. After complete incision, the entire gut was taken onto a watch glass with 10 mM phosphate buffer and minced with the help of sterile micro pestle (Vasanthakumar *et al*., 2009).

Metagenomic DNA isolation - Qualitative and quantitative analysis

Metagenomic DNA was isolated from the gut of *E. vigintioctopunctata* using commercially available Quick DNA MiniPrep Kit (Zymo Research), as per the manufacturer's instructions wherein ZR BashingBead™ Lysis tubes (0.1 and 0.5 mm) were filled with bacterial cells weighing 50-100 mg in 200 µL PBS isotonic buffer. Then 750 µL BashingBead™ buffer was added to the tube and processed at a maximum speed in a bead beater fitted with a 2 mL tube holder for 5 min. The ZR BashingBead™ lysis tube was centrifuged in a microcentrifuge at ≥10,000 x g for 1 min. Then supernatant (400 µL) was transferred to a Zymo-Spin™ III-F filter in a collection tube and centrifuged at 8,000 x g for 1 min. In collection tube from the previous step, 1200 µL genomic lysis buffer was added. Then 800 µL mixture from previous step was transferred to a Zymo-Spin™ II-CR column in a collection tube and centrifuged at 10,000 x g for 1 min. The flow-through from the collection tube was discarded and the preceding step was repeated. DNA pre-wash buffer (200 µL) was added to Zymo-Spin™ II-CR column in a new collection tube and centrifuged at 10,000 x g for 1 min. Then DNA wash buffer (500 µL) was added to ZymoSpin™ II-CR column and centrifuged at 10,000 x g for 1 min. The Zymo-

Spin™ II-CR column was then transferred to a clean 1.5 mL microcentrifuge tube and 100 µL (35 µL minimum) DNA elution buffer was added directly to the column matrix. This was then centrifuged at 10,000 x g for 30 sec to elute DNA (Lakoro *et al*., 2023). The quality of extracted metagenomic DNA sample was checked in NanoDrop by determining A260/280 ratio.

Library preparation and sequencing

Preparation of 2 x 300 MiSeq amplicon libraries using Nextera XT index kit (Illumina Inc.) in which primers: 16S rRNA F - GCCTACGGGNGGCWGCAG and 16S rRNA R - ACTACHVGGGTATCTA ATCC, with a sequencing adapter at end for the amplification of bacterial specific region, were designed and synthesized at Eurofins Genomics India Pvt. Ltd. (Bangalore). This step uses PCR to amplify the template out of a DNA sample using the region of interest specific primers with overhang adapters attached (Illumina CA, USA). First-round polymerase chain reaction (PCR) amplification was performed in a volume of 25 µL with the following reaction components: 2.5 µL of 50 ng genome DNA, 5 µL of 10 µM primer F, 5 µL of 10 µM primer R, 12.5 µL of 2 x KAPA HiFi HotStart Ready Mix (KAPA Biosystems, USA). PCR cycling parameters were 95°C for 3 min, followed by 25 amplification cycles of 95°C for 30 sec., 55°C for 30 sec, and 72°C for 30 sec, with 5 min final extension at 72°C. The purification of 16S V3 and V4 amplicon, away from free primers and primer dimer species, was carried out using AMPure XP beads. In order to add indices and adapter sequences, second-round tailed PCR amplification was done under the following conditions: 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec., 55°C for 30 sec., and 72°C for 30 sec., with a 5 min final extension at 72°C. The reaction system (50 µL) comprised of 5 µL products of first-round PCR, 5 µL Nextera XT index primer 1 (N7xx), 5 µL Nextera XT index primer 2 (S5xx), 25 µL 2 x KAPA HiFi Hot Start Ready Mix (KAPA Biosystems, USA) and 10 µL PCR grade water (Dudek *et al*., 2017). Then 3 µL PCR product was resolved on 1.2% agarose gel at 120 V approximately for 60 min (Fig. 1).

The amplified library was analysed on 4200 tape station system (Agilent Technologies) using D1000 screen tape as per the manufacturer instructions (Fig. 2). After obtaining the mean peak size from tape station profile, the libraries were loaded onto MiSeq at appropriate concentration (10-20 pM) for cluster generation and sequencing. Paired-end sequencing allows the template fragments to be sequenced in both the forward and reverse directions on MiSeq. The kit reagents were used in binding the samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of forward strands after re-synthesis of reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of fragment.

Statistical and bioinformatics analysis

A total of 1,69,846 raw reads were generated to survey bacterial communities. The raw data was first analysed using Trimmomatic (version 0.38) which is a flexible read trimming tool for Illumina NGS data to remove adapter sequences, ambiguous reads and low quality sequences along with a sliding window of 10 bp and a minimum length of 100 bp (Bolger *et al.,* 2014). The high-quality reads of each sample were spliced through overlap by FLASH Fast Length Adjustment of SHort reads) (version 1.2.11), an accurate, fast tool to merge paired-end reads from fragments that are shorter than twice the length of reads (Magoc *et al.,* 2011). OTUs were picked based on sequence similarity within the reads and a representative sequence for 16S bacteria from each OTU against Greengenes database (version 13_8) (McDonald *et al.,* 2012). OTU were assigned using reference databases and diversity metrics for each sample was calculated and types of communities were compared.

RESULTS AND DISCUSSION

The composition and relative abundance of gut bacterial communities of 4th instar larva of *Epilachna vigintioctopunctata* were analysed directly from the fields of *S. melongena* by Illumina MiSeq. This

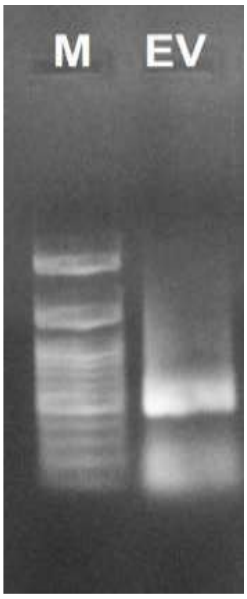


Fig. 1: Quality check on agarose gel of the PCR product of *Epilachna viginti-octopunctata* (EV). Here M stands for molecular marker (1 kb)

study revealed 1,69,846 raw reads. Cluster analysis showed a total of 553 OTUs, including 19 phyla, 52 classes, 84 orders, 159 families, 240 genera, and 271 species. The steep slope of rarefaction curve (Fig. 3), a plot revealing the number of species as a function of the number of sample, indicated that a large fraction of species-diversity remains yet to be discovered.

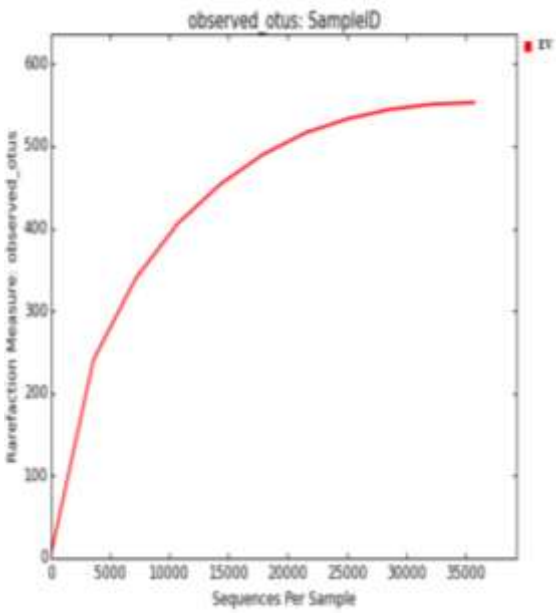
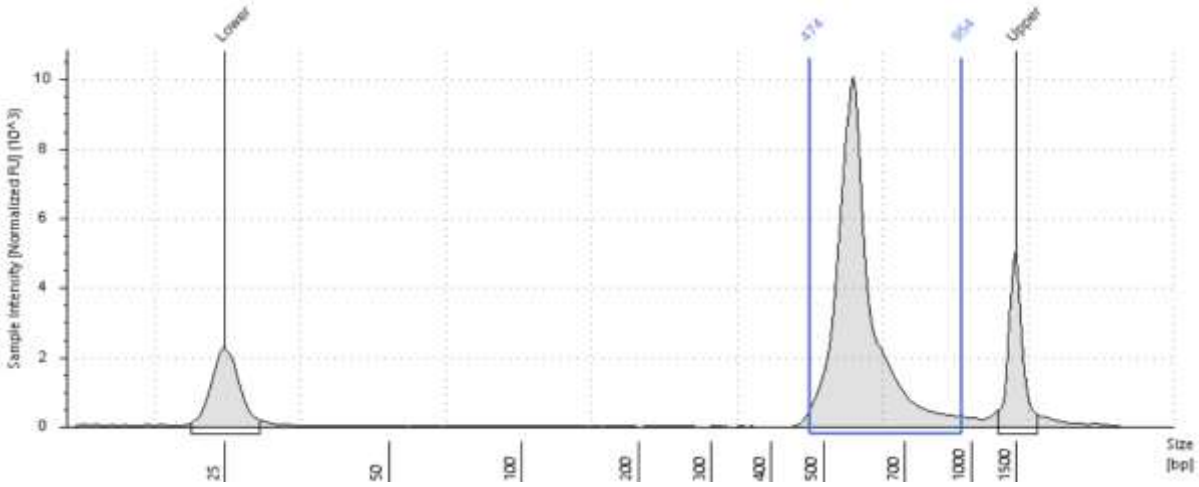


Fig. 3: Rarefaction curve is a plot of the number of species as a function of the number of sample. On the left, the steep slope indicates that a large fraction of the species diversity remains to be discovered. The vertical axis (Y axis) displays the diversity of the community while horizontal axis (X axis) displays the number of sequences considered in diversity calculation.



From (bp)	To (bp)	Average size (bp)	Conc. (ng μL^{-1})	Region molarity (nmolL^{-1})	% of total
474	954	596	32.0	83.9	92.69

Fig. 2: Tape-Station profile of library using D1000 Screen-Tape which was designed for the separation and analysis of DNA fragments from 35 to 1000 bp. A ladder was included with each run. The ladder contained two internal standards (lower and upper markers) to align the ladder data with samples to determine sizing. A region was set and is indicated by the vertical blue lines on either side of the library trace.

At phyla level, Proteobacteria was the most dominant phylum having an absolute count of 32095 and sharing a percentage of 89.76. This was followed by phylla Bacteroidetes, Firmicutes,

Actinobacteria and Chloroflexi with absolute counts of 1230, 1213, 856 and 125 sharing a percentage of 3.44, 3.39, 2.39 and 0.34, respectively (Fig. 4, Table 1). These findings are consistent with those of Lu *et al.* (2019) who characterized the gut microbial diversity in *E. vigintioctopunctata* fed on *Solanum melongena* and *S. nigrum* by sequencing the V3-V4 hypervariable region of 16SrRNA gene using Illumina MiSeq system. They too reported the presence of same four phyla (Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria) in *E. vigintioctopunctata* gut samples.

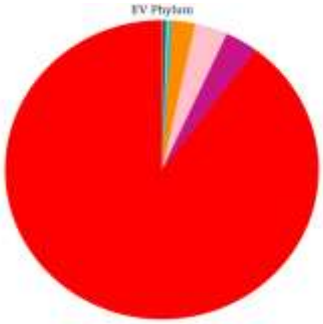


Fig. 4: Absolute abundance of each phylum within the bacterial community of the gut of *E. vigintioctopunctata*; The colours in the Pie chart correspond to those legends given in Table 1 which show the percentage of each phyla.

Table 1: The percentage abundance of phyla observed in the gut of *E. vigintioctopunctata*. The colours represent those present in the Pie chart of Fig. 4.

Legends	Taxonomy	Abundance
	k__Bacteria;p__Proteobacteria	89.77%
	k__Bacteria;p__Bacteroidetes	3.44%
	k__Bacteria;p__Firmicutes	3.39%
	k__Bacteria;p__Actinobacteria	2.39%
	k__Bacteria;p__Chloroflexi	0.35%
	k__Bacteria;p__Planctomycetes	0.16%
	k__Archaea;p__Euryarchaeota	0.15%
	k__Bacteria;p__Acidobacteria	0.11%
	k__Bacteria;p__Fusobacteria	0.07%
	k__Bacteria;p__Synergistetes	0.03%
	k__Bacteria;p__Gemmatimonadetes	0.03%
	k__Bacteria;p__Verrucomicrobia	0.03%
	k__Bacteria;p__TM7	0.02%
	k__Bacteria;p__Nitrospirae	0.02%
	k__Bacteria;p__Thermotogae	0.01%
	k__Bacteria;p__Chlorobi	0.01%
	k__Bacteria;p__OD1	0.01%
	k__Bacteria;p__Armatimonadetes	0.01%
	k__Bacteria;p__WS3	0.01%

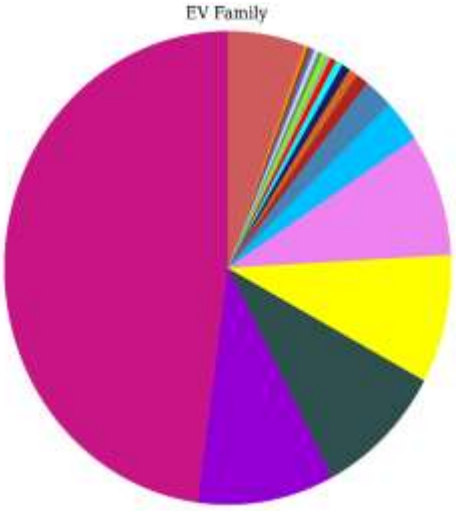


Fig. 5: Absolute abundance of each family in bacterial community of the gut of *E. vigintioctopunctata*. The colours in pie chart correspond to the legends in Table c below which shows the percentage of each phyla

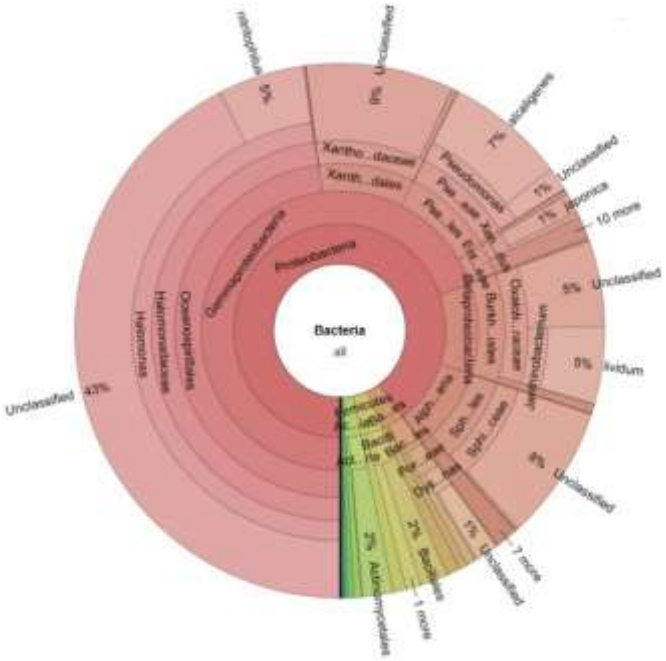


Fig. 6: Krona chart: It represents multi-layered Pie charts wherein hierarchial data can be viewed at multiple levels to visualize the abundance at any desired taxonomic level.

Table 2: The percentage abundance of each phyla in the gut of *E. vigintioctopunctata*. The colours represent those present in the Pie chart (Fig. 5)

EV Family legend

Legends	Taxonomy	Abundance
	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae	47.83%
	k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae	9.89%
	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Xanthomonadaceae	9.37%
	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae	8.77%
	k_Bacteria;p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae	8.35%
	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae	2.93%
	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae	2.11%
	k_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae	0.9%
	k_Bacteria;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae	0.65%
	k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae	0.61%
	k_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae	0.51%
	k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae	0.44%
	k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae	0.35%
	k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae	0.31%
	k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae	0.31%
	k_Bacteria;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae	0.3%
	k_Bacteria;p_Proteobacteria;c_Alphaproteobacteria;o_Unclassified;f_Unclassified	0.28%
	k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Propionibacteriaceae	0.25%
	k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Brevibacteriaceae	0.25%
	Others	5.57%

The study further revealed that the most dominant family was Halomonadaceae with abundance of 47.83%, which constitutes a phylogenetic lineage within the class Gammaproteobacteria and are mostly halophilic bacteria as per the 16S rRNA gene sequence analysis. This was followed by Oxalobacteraceae (Fig. 5, Table 2) with a percentage abundance of 9.89 in accordance with Noël Grégoire *et al.* (2021) showing the presence of Enterococcaceae and Oxalobacteraceae as major bacterial families in gut samples of *Galleria mellonella* (Lepidoptera: Pyralidae) exposed to polyethylene diet. Further, a Krona chart was constructed in order to visualize both the most abundant organisms and their most specific classifications (Fig. 6). It clearly showed Proteobacteria as dominant phylum while Gammaproteobacteria as the most dominant class with an abundance of 69.44%, followed by Betaproteobacteria and Alphaproteobacteria (Fig. 5, Table b).

Insect gut is colonized by a wide range microflora (Dillon *et al.*, 2004) including transient commensals (Hammer *et al.*, 2017) as well as obligate and facultative symbionts (Fisher *et al.*, 2017). The symbiotic association with microbes (especially bacteria) directly influences the biology of insect host and hence aids in the evolution of both host and symbiont (Douglas, 2015). The gut microbes assist the insect-host with metabolic benefits by providing vitamins and digestive enzymes that help in food digestion (Anand *et al.*, 2010). The gut microbes, in turn, safeguard the host from other pathogenic infection and also assists in detoxification of pesticides (Kikuchi *et al.*, 2012; Xia *et al.*, 2017). Metagenomics technique allows direct isolation of DNA from environmental samples in which a composite group of genomes is acquired from different organisms (Handelsman, 2004). This method is a culture independent technique. Kounatidis *et al.* (2009) employed both culture-dependent and culture-independent techniques and discovered the link between *Acetobacter tropicalis* and *B. oleae* successfully.

Hanwen *et al.* (2021) while working on the gut microbiome of *E. vigintioctopunctata*, by using 16S rRNA sequences derived from the adults fed on two distinct host plants *viz. S. nigrum* and *S.*

melongena, reported Proteobacteria as the most dominant phylum. This study analysed the diversity of gut microbiota of field-caught *E. vigintioctopunctata* larvae sampled in Karnataka based on sequencing of 16S rRNA genes extracted from metagenomic DNA, providing novel information regarding the bacterial diversity of *H. vigintioctopunctata*. It supports the view that the bacterial communities are dominated by a few taxa and an unclassified genus is dominant in the gut of *H.v igintioctopunctata*.

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