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Significant variations of bacterial communities among the developmental stages of *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae)

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The yellow stemborer, *Scirpophaga incertulas*, is a monophagous pest of rice, attacking the crop from its vegetative to reproductive stages. Microorganisms are crucial in influencing the insect's life cycle, evolution, and ecology, presenting an avenue for understanding and improving management strategies. Present research employed advanced next-generation sequencing technology to investigate the microbiota of *S. incertulas*, a previously unexplored area for developmental stage associated microbial diversity. The study used 16 S rRNA V3–V4 region amplicon sequencing to determine the diversity of bacteria associated with different developmental stages of *S. incertulas*. Taxonomically, bacterial communities were classified into 25 phyla, encompassing 46 classes, 101 orders, 197 families, and 364 genera. The major phyla identified were Proteobacteria (39%), Firmicutes (39%), Actinobacteria (11%), and Bacteroidetes (7%), with Proteobacteria being the most predominant across all developmental stages except the larval stage, where Firmicutes took precedence. Moraxellaceae, Bacillaceae, Xanthomonadaceae, Sphingobacteriaceae, and Flavobacteriaceae were predominant families across all the developmental stages. However, in the egg and adult stages, the abundance of Bacillaceae was notably lower, whereas Prevotellaceae found significantly higher in adult stages. Dominant genera across all stages included *Acinetobacter*, *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Pseudomonas*. The result showed that the highest number of Operational Taxonomic Units (OTUs) were in the larval stage (426 OTUs), the lowest in adults (251 OTUs), and the egg stage (254 OTUs). This suggests that the microbiota may play a role in the growth and development of *S. incertulas*. The predicted functional assessment of the associated *S. incertulas* microbiota revealed that the microbiota primarily participated in metabolic pathways, secondary metabolite biosynthesis, energy metabolism, signaling, and cellular processes. Our findings shed light on the significant variations in the microbial community and their predicted functions present in *S. incertulas* across developmental stages. The present study findings will help in developing novel microbiota-based management strategies.

Keywords Cereals, Insects, Microbes, Molecular, Moraxellaceae, Proteobacteria

The yellow stem borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae), is a notorious pest inflicting substantial losses on global rice crop production[1]. According to reports, it is Asia's number one rice pest, particularly in India[2]. The yellow stem borer infests rice crops at every growth stage, leading to yield losses varying between 10 and 90%, depending on the degree of infestation level[3–5]. It produces "dead hearts" in the vegetative

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stage of crop growth and "white ears" in the reproductive stage. The pest's cryptic nature, high fecundity, and wide adaptation to various environmental circumstances result in huge yield losses and necessitate specific pest management strategies. *S. incertulas* infests rice crops from egg to adult, with adults laying their eggs on rice leaves. The term "stem borer" denotes the larvae penetrating and tunneling into the stem of the rice plant, harming and weakening plants by feeding on the inner stem tissues. After a few weeks of feeding and growing inside the stem, the larval stages pupate within the rice plant. When the yellow stem borer completes pupation, it transforms into an adult moth and flies across fields[6,7].

Insect owns diverse microorganisms within their gut, hemocoel, exoskeleton, and cells, which play essential roles in their physiology and ecology[8]. Like many insect species, *S. incertulas* harbors various microbial communities that contribute to nutrient acquisition, pathogen defense, detoxification of plant secondary metabolites[9–11]. These symbiotic microbes influence the insect's immune responses, metabolism, and ability to exploit host plants effectively, thereby enhancing digestive efficiency, modulating host-plant interactions, and facilitating pest survival and adaptability[12,13]. Furthermore, microbiota composition varies across developmental stages, influencing susceptibility to pathogens and presenting opportunities for targeted microbial-based pest control strategies[14].

Environmental factors also affect microbiota dynamics, consequently impacting pest populations and their interactions with rice crops[9–11]. Study of developmental stage associated microbiota provides the crucial informations in several aspects of the host's life cycle such as development, evolutionary progress and nutritional needs at different developmental stages[4,9–11]. Several studies indicate insect gut microbiota changes significantly from larval to adult stages. For instance, in the brown planthopper, *Nilaparvata lugens* microbial composition shifts across developmental stages suggest that different developmental stages have their own gut microbiome[12,13]. In the Oriental fruit fly (*Bactrocera dorsalis*), microbiota composition has been linked to genes involved in immune responses, suggesting its role in the insect's adaptation[14]. Moreover, evidence indicates that microbiota can be conserved across life stages, implying co-evolution with the host[12]. To understand microbiota composition at different growth and developmental stages of insects is essential to identify the types of microbes present and know their responsive functions. This helps us gain insights into the specific bacteria for that stage, which helpful in devising strategies for insect pest management.

In the past, identifying bacteria involved isolating and culturing them and analyzing their physical traits, physiological requirements, and dietary needs. Researchers estimate that most bacteria found in animal gut and ambient samples are non-cultivable. However, the percentage of cultivable bacteria in insects like lepidopterans may differ. The insect's developmental stage and its external environment influence the gut microbiome composition[15].

High-throughput technologies have made significant progress in microbial community research in recent years. Two commonly used approaches for studying community structure are shotgun metagenomic sequencing and 16 S rRNA gene profiling. The 16 S rRNA gene has nine hypervariable regions, each of which is distinct in terms of its position, length, and taxonomic differentiation. The use of the hypervariable region V3–V4 facilitated the identification of bacterial species associated with insects. Ribosomal RNA genes possess characteristics that make them the most effective tool for phylogenetic investigations. These genes are highly conserved, meaning they remain mostly unchanged across time while still exhibiting variability in hypervariable regions[16–18]. At present, the 16 S rRNA gene is considered the best indicator for distinguishing the variety of microbial communities[16–18].

S. incertulas holds significance as an insect species in agriculture, yet there have been limited studies on its associated microbiota, with the majority relying on culture-dependent techniques[19] or molecular approaches of low resolution. High-throughput genome sequencing has not yet been used to describe the bacterial population associated with *S. incertulas*. As far as we know, there is a lack of available data on the microbiota composition across various developmental stages of *S. incertulas*. This study presents findings on bacterial communities linked to distinct developmental stages (egg, larval, pupal, and adult) of *S. incertulas* through 16 S rRNA gene metabarcoding on the Illumina HiSeq platform. Additionally, we conducted predictions regarding the functional metabolic activities of the bacterial community associated with *S. incertulas* developmental stages. This study aims to furnish data on bacterial diversity, the relative abundance of microbiota across various developmental stages, and the transmission of microbiota from immature stages to adult *S. incertulas*. We anticipate differences in the microbial communities hosted by *S. incertulas* among developmental stages, likely meeting the insect's needs at each metamorphic stage. Considering the adaptability of insect microbiota to different foods and the potential impact of the diet on insect microbiota, we also hypothesize an influence of host-plant species on the microbial community of the larval stage.

Results

Characterization of 16 S rRNA gene sequencing data

Illumina HiSeq technology was used to sequence the highly variable V3–V4 regions of the bacterial 16 S rRNA gene amplicon. The DNA extract was pooled from five samples and at least three replicates representing each developmental stage of *S. incertulas*. After analyzing the operational taxonomic units (OTUs) of the bacterial community, the larval stages had the most OTUs (426), followed by the pupal stage (289) (Fig. 1A). The OTU numbers across developmental stages indicate the complexity of the bacterial population associated with the *S. incertulas* stage. The Venn diagram illustrates the relationships between the OTUs, revealing that all developmental stages in *S. incertulas* shared only 51 OTUs (Fig. 1A). Based on the core microbiota distribution, the associated microbiota mainly comprised members of the phyla Proteobacteria (39%), Firmicutes (39%), Actinobacteria (11%), Bacteroidetes (7%), and others (4%) (Fig. 1B). The diversity indices (Fig. 2) reveal that the bacterial community diversity changed as *S. incertulas* progressed through its developmental stages. Various alpha diversity indices, including Ace, Chao1, Shannon, and Simpson, were analyzed, and results were compared

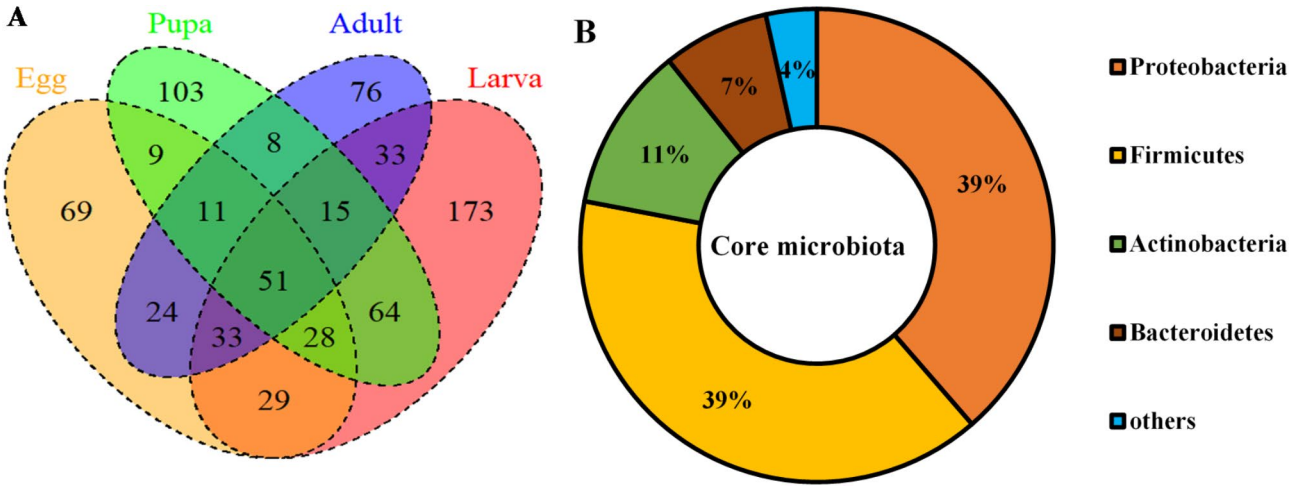

Fig. 1. The phyla percentage distribution of the 51 common strain-level shared OTUs across all developmental stages (**A**, **B**).

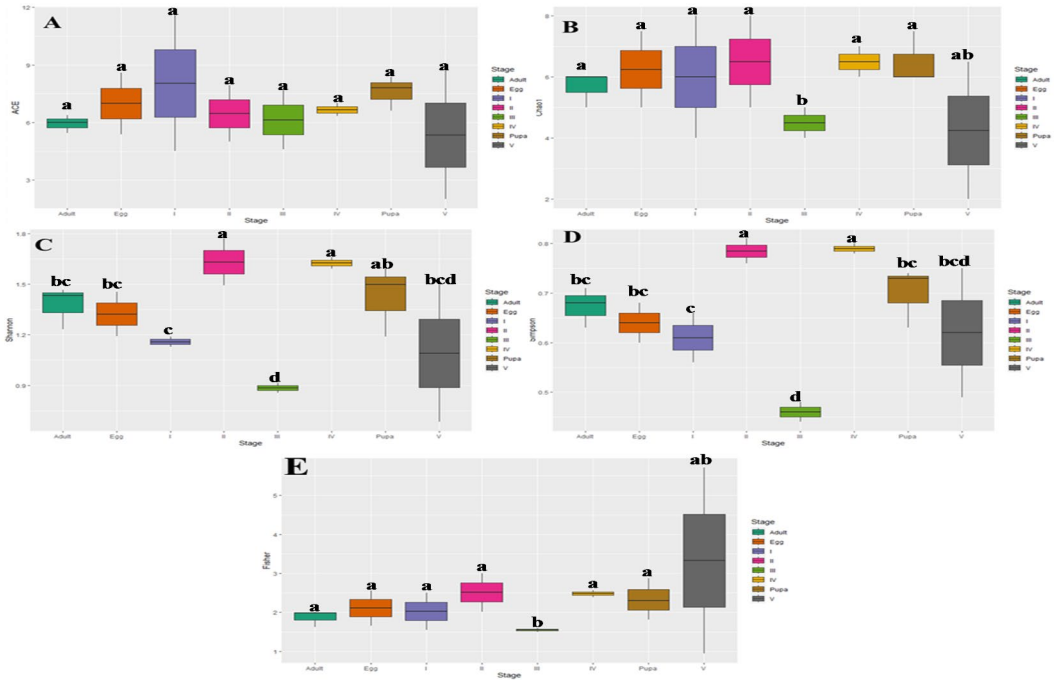

Fig. 2. A box plot illustrating the bacterial community's alpha diversity indices (**A**) ACE index; (**B**) Chao1 index; (**C**) Shannon index; (**D**) Simpson index; and (**E**) Fisher index) at different developmental stages of *N. lugens*. The different letters (a, b, c, d) on box plots indicate a significant difference ($p < 0.05$) and the same letters indicate no significant difference ($p > 0.05$) among the developmental stages using the Kruskal-Wallis H test.

using the Kruskal-Wallis H test ($p < 0.05$) (Fig. 2). Among the stages, the Ace indices were non-significant, with the highest Ace in the pupal stage (35.71%) and the lowest in the 5th instar (25.92%) ($p > 0.05$) (Fig. 2A). The Shannon and Simpson species diversity indices were highest in the egg stage (2.84%) and (0.91%), respectively, and the lowest was found in the 5th larval stage, i.e., Shannon (1.98%) and Simpson (0.78%) ($p < 0.05$) (Fig. 2C and D).

Microbiota taxonomic composition across developmental stages

A taxonomic study of the associated microbiota revealed that, after excluding unclassified and unassigned bacteria, the various stages of *S. incertulas* included 25 bacterial phyla divided into 46 classes, 101 orders, 197 families, and 364 genera. Each developmental stage had its own microbial community, as shown in the

taxonomic comparison (Fig. 3). Across all stages, only a few phyla -Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes—were consistently dominant (Fig. 3). Proteobacteria was the most abundant phylum in all stages except the larval stage, where Firmicutes predominated. Firmicutes had a range of 18.19% (adult stage) to 47.5% (larval stage), while Proteobacteria had a range of 34.8% (larval stage) to 46.21% (pupal stage) (Fig. 3). The bacterial community was slightly more abundant in late larval stages compared to earlier developmental stages, as shown in the family-level heat map of the OTUs (Fig. 4). The most prevalent genera were *Acinetobacter* (10%), *Bacillus* (5%), *Lactobacillus* (5%), *Enterococcus* (5%), and *Pseudomonas* (5%). Additionally, bacterial genera like *Klebsiella*, *Citrobacter*, *Bacteroides*, and *Flavobacteria* were present in all developmental stages (Supplementary Fig. 1).

Structures of bacterial communities throughout different developmental stages

The analysis showed that the proportion of phyla Firmicutes and Proteobacteria across developmental stages were the primary cause of differences in community architecture (Fig. 3). Further analysis of the average similarity between developmental stages revealed that *Bacillaceae* under the phylum Firmicutes were significantly less common during the egg stage as compared with other stages. The predominant families in the egg stage were *Xanthomonadaceae*, *Spingobacteriaceae*, and *Flavobacteriaceae* (Fig. 5). Comparing the egg stage with the pupal and adult stages, *Moraxellaceae*, *Xanthomonadaceae*, *Spingobacteriaceae*, and *Flavobacteriaceae* were much more abundant (Supplementary Fig. 2). In the adult stage, *Bacillaceae* numbers were much lower than in all other stages. The families *Moraxellaceae*, *Erysipelotrichaceae*, and *Prevotellaceae* were significantly more abundant in adults compared to other stages (Fig. 6). In the pupal stage, the family *Paenibacillaceae* differed significantly from all other stages (Supplementary Fig. 3). Genera such as *Acinetobacter*, *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Pseudomonas* are dominant at all stages, whereas genera like *Bacteroides* and *Deinococcus* were found to be significantly more abundant in males and females but less abundant in other developmental stages (Supplementary Fig. 1).

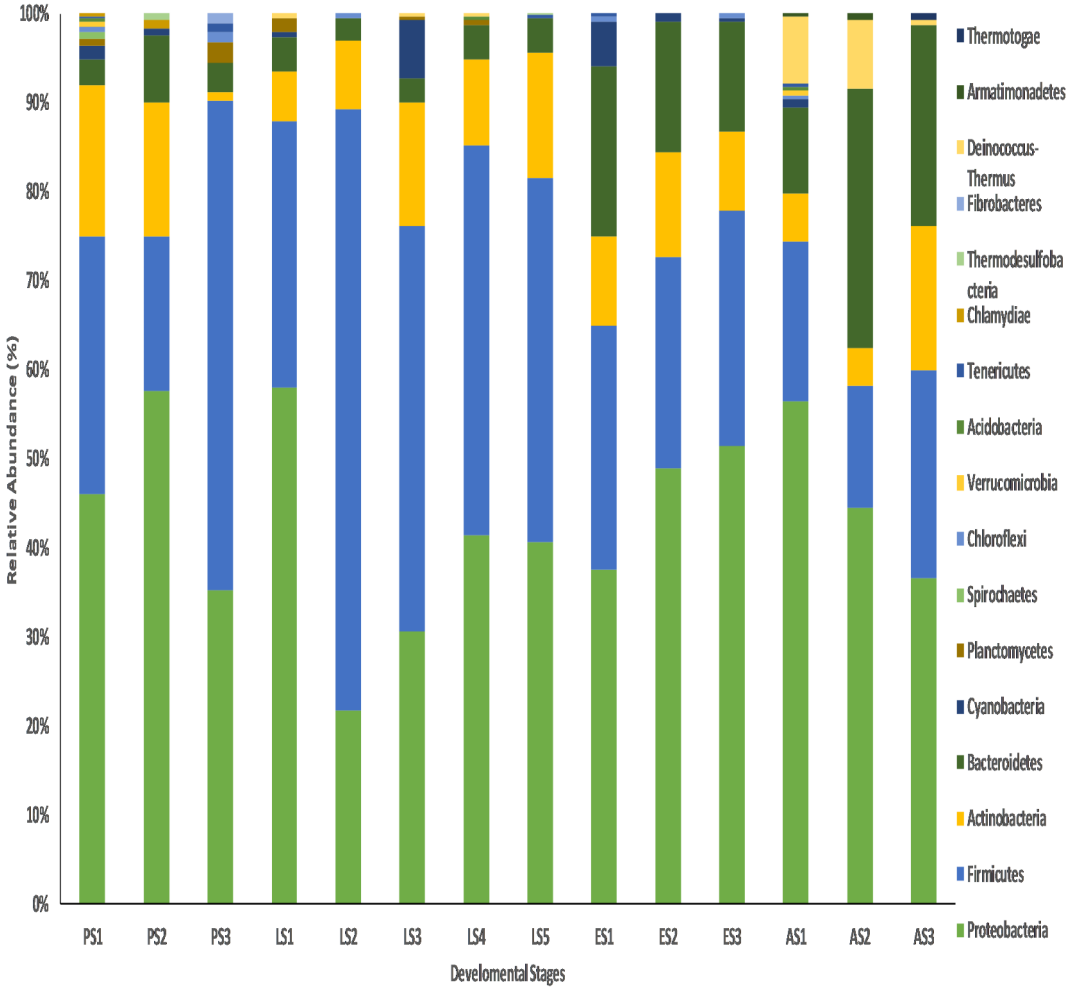

Fig. 3. The phylum-level distribution and relative abundance (%) of the bacterial population associated with *Scirpophaga incertulas* at various developmental stages. ES: egg stage; LS: larval stage (1–5th instars); PS: pupal stage; AS: adult stage.

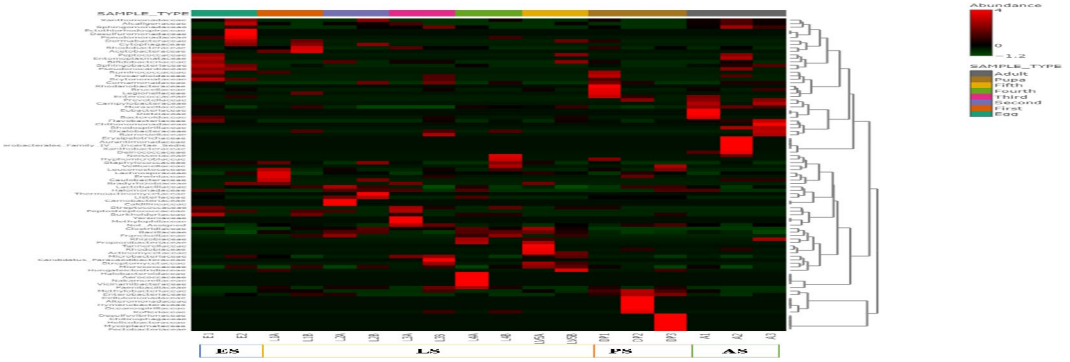

Fig. 4. Heatmap illustrates the family-level relative abundance of 16 S rRNA gene OTUs at various developmental stages. The developmental stages of *S. incertulas* shown are the egg stage, larval stage, pupa stage, and adult stage (ES, LS, PS, and AS).

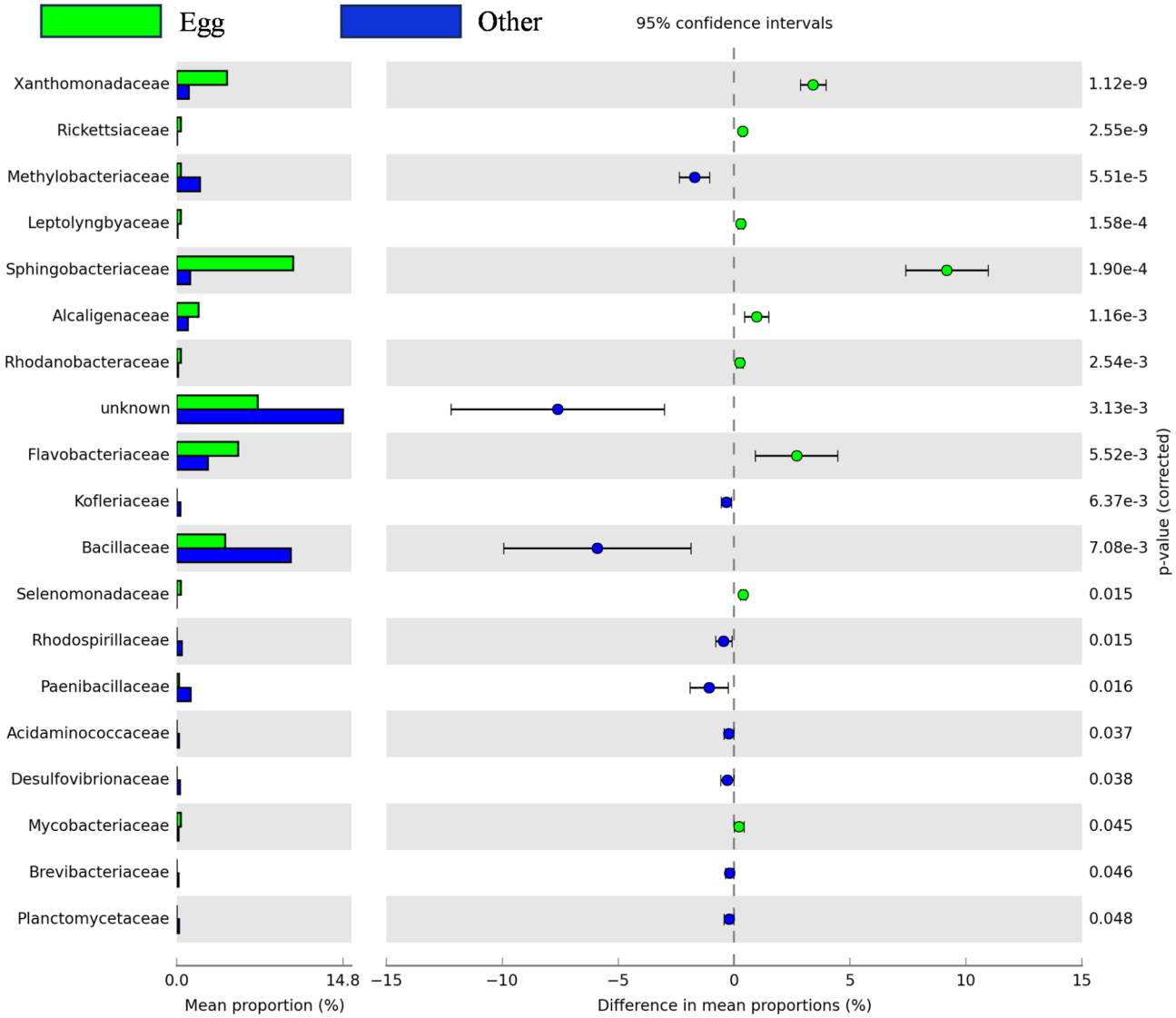

Fig. 5. The average difference of bacterial families among the egg and other stages of development with p values < 0.05 (Welch's t-test, FDR adjusted).

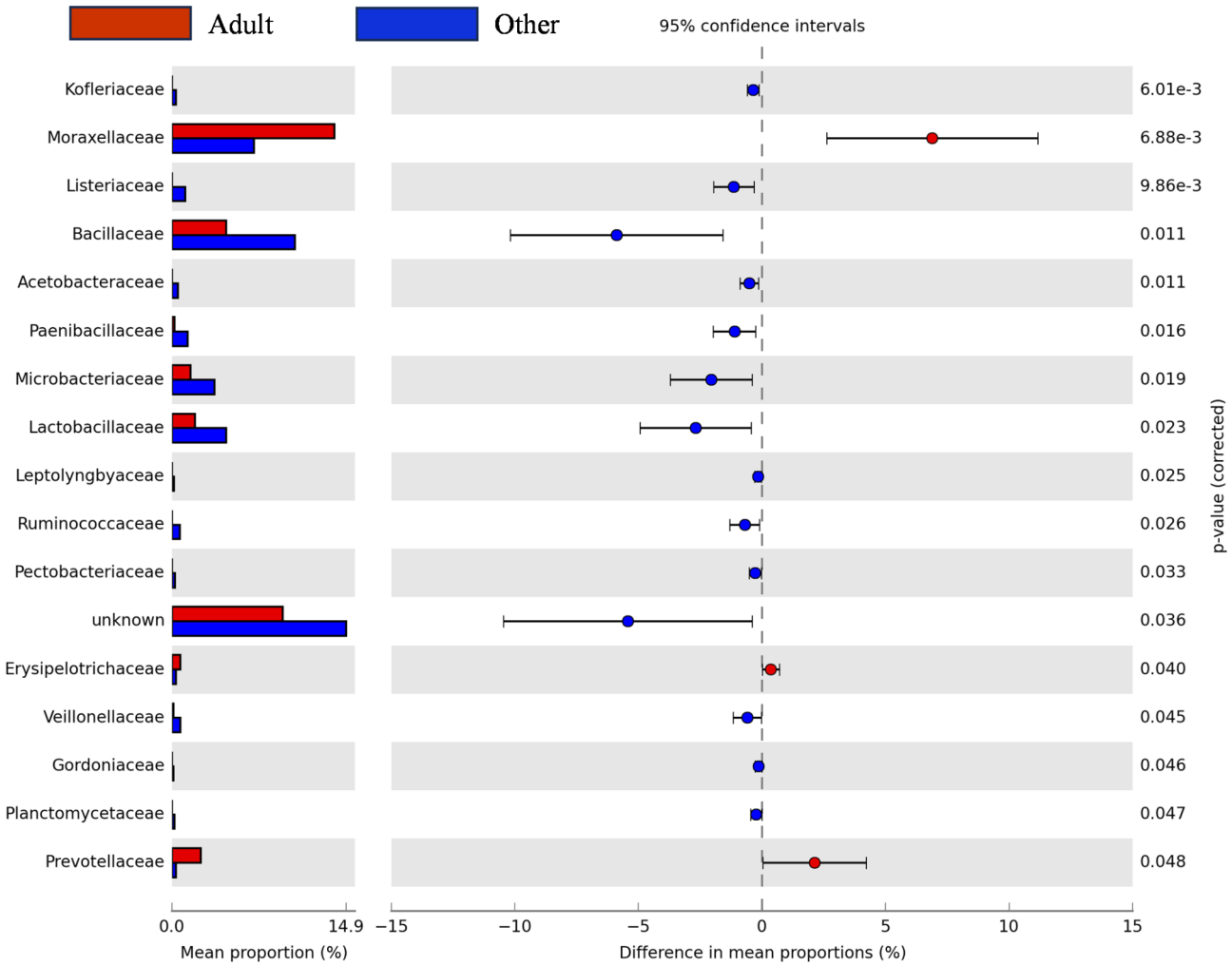

Fig. 6. The average difference of bacterial families among the adult and other stages of development with p values < 0.05 (Welch's t-test, FDR adjusted).

Prediction of functional pathways across developmental stages

Based on functional orthologs in the predicted metagenomes of *S. incertulas* developmental stages, 4259 categories of metabolic processes were identified. The analysis revealed genes related to metabolic pathways, secondary metabolites, biosynthesis, energy metabolism, ABC transporters, thermogenesis, protein digestion and absorption, circadian entrainment, signaling, and cellular processes were abundant in bacterial communities linked to developmental stages (Supplementary Fig. 4). Bacterial endosymbionts were involved in membrane transport, signal transduction, and transcription through functional pathways. Major Facilitator Superfamily (MFS) transporters respond to ion gradients by transporting small molecules; ABC transporters (ATP binding cassette), which can move small ions via ATP to macromolecules; and phosphotransferase systems (PTS), During transport, absorb carbohydrates and convert them into their corresponding phosphodiester. These are possible connections between these pathways. Similarly, endosymbionts are associated with the cAMP signaling system, which controls various physiological functions such as gene transcription, metabolism, secretion, muscle contraction, and calcium homeostasis.

Discussion

This study is the first comprehensive report of the Illumina sequencing and annotation of the bacterial population associated with every stage of *S. incertulas* development. Culture-based techniques have limitations, such as detecting only cultivable organisms, which results in missing a significant portion of microbial diversity. Additionally, culturing is time-consuming, and some species may not grow under standard conditions due to the selectivity of the chosen culture medium. These methods allow the identification of less than 1% of the insect's associated microbiota[33]. Here, we performed 16 S rRNA amplicon analysis using Illumina sequencing, which provides a comprehensive understanding of the diversity and evenness of bacterial species within a community by simultaneously studying thousands of DNA fragments. The present study's chosen amplicon lengths (250 bp), inclusion of several primer pairs, and recognition barcodes allowed us to analyze amplification biases, the

amount of sequencing error, and the correlation between paired-end reads, among other features[34,35]. But the short and long length of read data accuracy is true when what is the taxonomic group of interest[36].

We identified 364 genera from multiple bacterial communities in *S. incertulas* at different growth phases. Compared to the previous study from stripped stem borer, *Chilo suppressalis*, more taxa have been identified across various developmental stages of *S. incertulas*[37]. The current study validated the shifts in bacteria biodiversity that occurred while *S. incertulas* developed. Proteobacteria dominated Firmicutes in every developmental stage of *S. incertulas* except for the larval stage. The research of another Lepidoptera, including *Manduca sexta*, *Lymantria dispar*, *Bombyx mori*, *Grapholita molesta*, *Spodoptera littoralis*, and *Helicoverpa armigera*, was comparable to this one[37–40]. These differences reflect a new perspective on the coevolution of the gut symbiotic bacteria and the host, as they are dictated by the diet and behavioral characteristics of the host insects[41]. Proteobacteria and Firmicutes are two phyla that can break down complex polysaccharides enzymatically, aiding the nitrogen fixation process. The bacterial phylum and its corresponding host have a symbiotic relationship that may aid the host's growth, development, and reproduction ability. The gut microbiota significantly affected male fertility, as observed in *Spodoptera frugiperda*[42]. Lepidopterans' gut microbiomes are remarkably diverse, indicating that they do not depend on a stable, beneficial microbiome that is present in all subsequent generations. Additionally, this variation might point to the potential for a highly dynamic microbiome, which would allow their hosts to adapt to Lepidopteran bacterial symbionts in response to shifting environmental factors like abiotic condition shifts, food availability, and the threat of natural enemy attack[43].

The phylum Proteobacteria is said to be involved in the breakdown of carbohydrates such as starches and hemicellulose, as well as the breakdown of pectin and nitrogen. Much like the guts of numerous other invertebrate and vertebrate species, lepidopteran guts are residences of highly active microorganisms like bacteria, archaea, and fungi[43,49]. Zhang et al.[49] found that the Enterobacteriaceae, Bacillaceae, and Pseudomonadaceae families were the most prevalent after scanning thirty lepidopteran species. A study on taxonomy revealed that Actinobacteria was the most common phylum in lepidopteran insects, followed by Firmicutes and Proteobacteria; *Acinetobacter*, *Pseudomonas*, *Pantoea*, *Enterobacter*, *Clostridium*, and *Enterococcus* were the most common genus-level bacteria[50,51]. It has been proposed that firmicutes participate in the assimilation of energy from the diet and may impact development[7], undermine plant defenses against herbivores[52,53], and boost insecticidal and herbivore defenses[54]. In the species *Eisenia fetida*, Actinobacteria have been reported to assist in antibiotic resistance[55]. Furthermore, as reported in *S. frugiperda*, nutritional and environmental variables can potentially alter the composition of the bacterial community[56–59].

Previous studies have demonstrated bacterial diversity consistent with our findings. For example, isolates of *Pseudomonas* and *Acinetobacter* from various insects, including *D. rhizophagus*, *B. mori*, and *Saperda vestita*, exhibit amylolytic, cellulolytic, xylanolytic, lipolytic, and esterase activity[44–46]. *Pseudomonas* species within insect guts can enzymatically degrade pesticides, thereby reducing their toxic effects[47,48]. Furthermore, *Acinetobacter* spp. have been found to break down complex organic molecules and xenobiotics, such as polycyclic aromatic hydrocarbons, phenol compounds, and polychlorinated compounds[74,75]. The ability of *Acinetobacter* spp. to induce mortality in *Spodoptera littoralis* larvae highlights its potential application in biological control strategies[76]. Notably, *Citrobacter* spp. in *S. inferens* can degrade biopolymers such as cellulose and xylan, which are vital for nutrient extraction from plant cell walls[45]. Additionally, members of the genera *Enterobacter* and *Klebsiella* are involved in digestive processes in *S. littoralis* larvae, aiding in carbohydrate metabolism and biomass degradation[77]. The elevated carbon-to-nitrogen percentage in leaves suggests that chewing insects struggle with insufficient nitrogen in their diet[60–62]. Because symbiotic bacteria fix nitrogen and transform it into nitrogen-containing molecules that are physiologically relevant, they may be advantageous. It is well known that rhizobacteria attached to plant roots convert molecular dinitrogen to ammonium. Termites and other insects carry microorganisms that can change nitrogen dioxide to ammonia. After being absorbed by gut endosymbionts, this ammonia is converted into vitamins and amino acids essential for insect development[63].

Our results demonstrate significant shifts in microbial composition across the developmental stages of *S. incertulas*, with variations in microbial families and genera influencing metabolic processes, ecological roles, and interactions with rice plants. At the egg stage, the microbiome was dominated by families such as Xanthomonadaceae, Sphingobacteriaceae, and Flavobacteriaceae, which are involved in nutrient cycling and polysaccharide degradation[64,65]. These families likely to facilitate early microbiome colonization and nutrient acquisition. Specifically, *Xanthomonas* species may alter the feeding behavior of rice pests, masking plant defenses, and making the plants susceptible to pest thereby progressing the pest survival and development in rice fields[64]. The reduced abundance of Bacillaceae observed in egg and adults stage suggests a decreased need for these functions (nitrogen fixation and organic matter degradation) during the non-feeding stage. As *S. incertulas* progresses to the adult stage, microbial composition shifts towards families such as Moraxellaceae, Erysipelotrichaceae, and Prevotellaceae, known for their roles in energy metabolism and host-microbe interactions[15,16,66]. The increase in Moraxellaceae, particularly *Acinetobacter*, which is involved in nitrogen metabolism, reflects the heightened metabolic demands of oviposition[65]. These shifts indicate the adult stage's need for enhanced metabolic processing to support reproduction and energy storage[66].

Genera like *Acinetobacter*, *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Pseudomonas* were prevalent across all stages, highlighting their broad functional roles. *Bacillus* species enhancing growth and pathogen resistance[15]. *Lactobacillus* and *Enterococcus* play key roles in gut health, supporting digestion and immune responses, which are vital for survival in challenging rice field environments[15,65]. *Pseudomonas*, known for its antimicrobial properties, aids in pathogen defense and nutrient acquisition[47,48]. The genera *Bacteroides* and *Deinococcus* were more abundant in adult males and females, suggesting a role in sexual dimorphism and reproductive physiology. *Bacteroides*, involved in polysaccharide degradation, may enhance energy extraction during reproduction, while *Deinococcus* helps the insect cope with environmental stresses like desiccation, improving survival in rice fields[67].

The outcomes of this study, which are based on the function-based prediction of the bacterial ecosystem associated with various developmental stages of *S. incertulas*, demonstrate that the bacterial community is involved in various processes, including the synthesis of proteins and the metabolism of starches and amino acids. In contrast, these metabolic processes may be compromised in insects whose diets are low in nitrogen. Several studies on Lepidoptera suggest that the microbiome of Lepidoptera has various effects on its hosts, so it's crucial for the health and fitness of the host; the microbiome diversity of insects affects their developmental stages and feeding behavior[38,70–73]. Further investigation is necessary to understand the underlying components of these interactions fully. The chosen amplicon lengths (250 bp) are also acceptable. However, the increased length of reads through PacBio and Nanopore are more useful for metagenomic classification despite being more error-prone than Illumina reads[36].

In conclusion, present study highlights the importance and changes of bacterial communities in shaping the growth and development of major rice insect pest i.e., *S. incertulas*. Study found that the dominant bacterial communities in *S. incertulas* changes across different developmental stages. Bacterial families like Bacillaceae, Moraxellaceae, Xanthomonadaceae, and Flavobacteriaceae, along with genera such as *Acinetobacter*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Bacteroides*, and *Deinococcus*, play crucial roles in nutrient cycling, detoxification, immune modulation, and energy metabolism across developmental stages of the pest. Given their role in nutrient assimilation and insecticide degradation, manipulating these microbial communities offers promising avenues for sustainable pest control strategies. Future research should focus on functional validation of these microbial interactions to develop microbiome-based pest management techniques, ultimately improving rice crop protection and integrated pest management efforts.

Materials and methods

Collecting various life stages of *S. incertulas*

The initial larval stage of *S. incertulas* was collected from the TN1 rice variety at the ICAR-National Rice Research Institute research farm in Cuttack, India (20°26'54"N; 85°56'25"E) in September 2022. The specimens were then transported to a net house with conditions set at 27 ± 1 °C temperature, 70 ± 5% relative humidity, and a 14-hour light: 10-hour dark photoperiod for rearing. The insects were released onto 60-day-old potted TN1 plants for oviposition, with freshly emerged adults placed on fresh 60-day-old potted plants. All developmental stages from the F1 progeny were collected separately, including the egg, all larval instars (1st to 5th instar), pupae, and adults. The samples of 2-days-old eggs, emerged larvae (1st to 5th instar), 2-days-old-pupae, and 1-day-old emerged adults (male and female) were collected and stored at -80 °C for DNA extraction and further 16 S rRNA amplicon sequencing.

Extraction of DNA, amplification of the targeted V3-V4 region, and sequencing

The whole midgut tissues of five individuals per replicate were dissected from 2nd to 5th instar larvae, pupae, and adults in a sterile environment. The whole body of eggs (100 eggs per replicate) was used for DNA extraction because of their small size. Thus, 3 replicates for the egg stage, 10 for the larval stage, 3 for the pupal stage, and 3 for the adult stage were used for sequencing. Samples collected from various life stages were surface sterilized for 60 s with a 0.1% mercuric chloride solution, and any remaining chemical was removed by washing them with sterilized distilled water[17–22]. The DNeasy Blood and Tissue Kit (Qiagen, Germany) was used to extract DNA from individual samples at various stages following the manufacturer's instructions. After that, NanoDrop-OneC (ThermoFisher, USA) was used to quantify the DNA. For characterization of bacterial communities, V3-V4 hypervariable regions of 16 S rRNA amplicon were amplified using the 341 F and 805R primers with added Illumina adaptor overhang sequences (16 S rRNA gene-specific sequences are underlined) (341 F: 5′-TCGT CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and (805R: 5′- GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′)[23]. Utilizing the NEBNext Q5 Hot Start HiFi PCR master mix (New England BioLabs) and the NEBNext Ultra II DNA Library Prep Kit (New England BioLabs), library preparation was carried out following the manufacturer's protocol, where PCR conditions included 30 s of first denaturation at 98 °C then 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. Ultrapure water (without DNA from samples) was used to exclude the possibility of false-positive PCR results as a negative control. All the amplicons were checked by electrophoresis in agarose gel (0.8%) and sequenced with the Illumina Hi Seq technology[17]. The NEBNext Multiplex Oligos Kit for Illumina (2 × 250 bp) was utilized. Utilizing the Illumina Hi Seq system (Hiseq Rapid SBS Kits v2) (2 × 250 bp paired-end reads) at NovelGene Technologies Pvt Ltd, Hyderabad, India, following the manufacturer running protocol (Illumina, Inc, San Diego, California U.S.A).

Assemble, metagenomics sequence annotation, and statistical analysis

Using FastQC software, raw sequences acquired for various development phases were examined for GC distribution, base quality, and base composition, among other criteria[24]. The Quantitative Insights into Microbial Ecology pipeline (QIIME version 1.9.1) was utilized to process and analyze the raw sequences[25]. After eliminating the low-quality read, paired Illumina reads were quality-trimmed and combined using the FLASH program (version 1.2.11) to produce the consensus sequences[26]. Additionally, chimera sequences were eliminated by applying the UCHIME algorithm, which was included in the VSEARCH program version 1.7.0[27]. Using the Uclust tool, version 1.2.22 in QIIME (version 1.9.1), the sequence reads of all samples were combined and grouped into operational taxonomic units (OTUs) based on their 97% sequence similarity[28]. PyNASTtool, using a threshold value of 80%, which targets the V3–V4 region of 16 S rRNA was used as the taxonomy assignment tool, and the taxonomy given to the sequences was determined by consulting the Silva database version 13.5[29]. For all downstream analyses, output biological observation matrix (BIOM) files were utilized. The MicrobiomeAnalyst web platform (https://www.microbiomeanalyt.ca/) and R (version 4.0.4) were used to

conduct the downstream analyses. The Chao1, Ace, Shannon, Simpson, and Fischer indices, as well as a relative abundance heatmap produced with the Bray-Curtis dissimilarity index approach, are included in the diversity study[30]. Box plots of different diversity indices such as ACE, Chao1, Shannon, and Simpson were plotted using a vegan package in R. The significant differences in alpha diversity were analyzed using the Kruskal-Wallis H test performed in SPSS (version 22.0). To determine which OTUs of *S. incertulas* were responsible for the observed differences, a similarity percentage analysis (SIMPER) of the unique and shared OTUs across the developmental phases of the organism was carried out using the PAST 3.25 software package[31]. The PICRUSt v. 1.1.4 program predicted metabolic functions from the 16 S rRNA sequence data against the KEGG orthology functional database[32]. Final SRAs (Sequence Read Findings) can be accessed under Bioproject accession ID PRJNA1034813 at the NCBI SRA database, https://www.ncbi.nlm.nih.gov/sra/PRJNA1034813.

Data availability

SRAs (Sequence Read Findings) can be accessed under Bioproject accession ID PRJNA1034813 at the NCBI SRA database, https://www.ncbi.nlm.nih.gov/sra/PRJNA1034813.

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Author contributions
GGPP, AM & SDM conceptualized the idea; GGPP, RMP, and AM collected the insect samples; JSC, BG, RMK and NP analyzed the results; NP, BG, and SDM wrote the initial draft; GGPP, RMP, KS, RMK and JSC wrote the final draft. All authors contributed to the article and approved the submitted version.

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