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SEX-DEPENDENT METAGENOMIC INSIGHTS INTO THE ENDOSYMBIOTIC BACTERIAL ECOLOGY OF *ZYGOGRAMMA BICOLORATA* PALLISTER (COLEOPTERA: CHRYSOMELIDAE)

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ABSTRACT:
Insects are among the most ecologically successful animal taxa and are known to harbour diverse endosymbiotic microorganisms, including bacteria, viruses, fungi, and protozoa, which can influence various aspects of host biology. This study investigates the bacterial endosymbiont communities associated with the parthenium beetle (*Zygogramma bicolorata* Pallister), a biocontrol agent for a weed, *Parthenium hysterophorus*. We hypothesized that *Z. bicolorata* harbours a diverse microbiota that may contribute to sex-specific physiological or ecological functions. Male and female beetles were subjected to 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform. Taxonomic profiling revealed notable differences in bacterial community composition between the sexes. Males were dominated by Proteobacteria (67.17%), followed by Firmicutes (15.26%), Cyanobacteria (6.39%), Bacteroidetes (6.30%), and Actinobacteria (4.14%). In contrast, females exhibited a marked predominance of Firmicutes (57.53%), with comparatively lower proportions of other phyla. These findings indicate that *Z. bicolorata* harbours a diverse and sex-dependent bacterial community, suggesting potential functional roles of endosymbionts in host development, physiology, or ecological adaptation.

KEYWORDS: Bacterial Diversity; 16s Metagenomics; Proteobacteria; Firmicutes; Parthenium beetle.

INTRODUCTION

The ecosystem serves as the essential foundation of ecology, comprising both the abiotic components of the environment and the biotic communities that interact within it [1]. Insects, as part of the biological community, engage with both the living organisms and the physical components of their environment. They are involved in nutrient recycling processes by decomposing leaf litter [2], woods [3], carrion [4], dung [5], as well in food Chains [6], pollination [7], soil aeration [8], environmental indicators [9], symbiotic relationships [10], disease vectors [11], cultural and economic roles [12], and to facilitating the spread of fungi [13]. In addition, insects are essential to the food web, particularly for vertebrates that consume them, such as reptiles. Insects offer significant advantages in terms of nutritional value. Their nutritional compositions are actually quite similar to those of the traditional animal foods [14]. Many insects have been eaten worldwide in many areas such as Lepidopterans, Orthopterans, Isopterans and Hymenopterans [15].

In many nutritional deficiencies of animals fed a diet consisting solely of insects have been documented in Amphibians, birds, and mammals [16-19]. The ecological significance of insects is underscored by their wide variety of forms, functions, behaviours, biomass, and interactions with their surroundings abiotic as well as biotic factors [20]. Biotic interactions are the influences that two organisms in a community have on each other in a non-trophic way this can occur at an interspecific or intra-specific level [21]. These interactions can result in either positive or negative effects and take a number of different forms such as predation [22- 23], symbiosis [24], competition [25] and parasitism [26].

Among the above, symbiosis is a mutualistic relationship between various insects and other organisms for their shared advantage. Various insects form symbiotic relationships for several reasons such as food [27], protection [28], and shelter [29], and even unintentionally [30]. A diverse array of organisms, including both prokaryotes and eukaryotes, exist within other cells as intracellular endosymbionts, this phenomenon is known as endosymbiosis, which has deeply impacted evolution and also governs the shape of the ecology and behaviour of the different species [31].

Endosymbiotic associations are widespread among invertebrate phyla, including Protozoa [32], Porifera [33], Cnidaria [34], Platyhelminthes [35], Nematoda [36], Mollusca [37], Annelida [38], Arthropoda [39], and Echinodermata [40]. Amongst the above, Arthropod is one of the most ancient, diverse, and ecologically dominant animal groups on the earth [41]. Besides, numerous species maintain symbiotic relationships with microbial partners [42]. These associations have been documented across several arthropod classes, including Crustacea [43], Myriapoda [44], Arachnida [45], and particularly Insecta [46], the most specious and extensively studied group within the phylum. Insect-associated endosymbionts contribute to a range of host physiological and ecological functions. These include nutritional

supplementation, immune modulation, protection against pathogens and parasitoids [47, 48], compensation for nutrient-deficient diets [49], and mediation of inter- and intraspecific communication and reproductive behaviors [50-51]. Similarly, Endosymbionts helps in the pheromone synthesis, digestion, detoxification and the providing essential amino acids and other metabolic compounds [52-53]. This multitasking role underscores the vital contribution of microbial symbionts to insect biology, reflecting a high degree of evolutionary dependence and co-adaptation.

Insect-associated microorganisms have the potential to assist insects in several ways such as offering protection [54], facilitating detoxification [55], providing nutrition [56-57], improving communication [58], and influencing the existence of other organisms [59]. Microorganisms can modulate plant defense mechanisms in ways that benefit their associated insect hosts [60-61]. In addition to these indirect effects, plant-associated bacteria may also influence the dynamics of insect–plant interactions, shaping ecological outcomes across trophic levels. Coleoptera (Beetles), the most diverse insect order, exhibit numerous symbiotic relationships that contribute significantly to their ecological adaptability and evolutionary success [62-64].

These microbial partnerships play vital roles in enhancing the nutritional physiology of herbivorous beetle species by facilitating digestion and nutrient acquisition [65-68]. Additionally, symbionts can confer defensive capabilities, such as protection against predators, parasites, and pathogenic microbes [69-70]. Together, these functions illustrate the integral role of microbial symbionts in supporting beetle diversification and ecological success.

This study explores the complex interactions between beetles and their associated microbial communities within Coleoptera. Numerous bacterial species associated with arthropods have been identified as either endosymbionts or entomopathogens [71-72]. Among these, *Zygogramma bicolorata*, a leaf beetle from the family Chrysomelidae, holds particular importance as a biocontrol agent, especially in the management of the invasive weed *Parthenium hysterophorus* [73]. Due to its efficacy, environmental safety, and cost-effectiveness, *Z. bicolorata* is considered one of the most ecologically sustainable options for biological control.

The primary objective of this study was to characterize the metagenomic profile of the bacterial community associated with adult *Z. bicolorata*. A significant proportion of environmental microbes are unculturable using conventional synthetic media, traditional culture-dependent methods offer only limited insight into microbial diversity. To overcome this limitation, we employed 16S rRNA gene sequencing using the Illumina platform to comprehensively assess the beetle's microbiome. The resulting data provide valuable insights into potential endosymbiotic relationships in *Z. bicolorata*, contributing to a better understanding of its reproductive biology and its effectiveness as a biocontrol agent.

2. MATERIALS AND METHODS

2.1 Samplings and DNA extraction

dults and larvae of *Zygogramma bicolorata* were collected from local agricultural fields in Amarkantak, India (22º 40'N, 81º 45' E). They were raised on a continuous supply of fresh-cut leaves from *Parthenium hysterophorus* in plastic Petri dishes (14.5 × 1.5 cm²). In a BOD incubator, these dishes were maintained under stable environmental conditions (25 ± 2ºC temperature, 65 ± 5% relative humidity, a photoperiod of 14 hours light and 10 hours dark) (REMI CHM-16 Plus). The wilted leaves were replaced daily with fresh ones at the same time. Adult males and females were placed together in the Petri dishes to mate until they naturally separated. After mating, the female was moved to a new Petri dish to observe daily egg-laying and larval hatching. The newly hatched larvae were kept in Petri dishes until they reached the fourth instar. Once they reached this stage, the larvae were transferred to a glass beaker (500 ml) filled with moist sand for pupation. The adult beetles emerged from the beakers after approximately 10±1 day. The 10-day-old adults were then selected from the stock culture for further analysis. Beetles were sterilized twice in 70% ethanol to remove surface-contaminating microbes and residual ethanol was removed with a single rinse in sterile milliQ water. For the whole beetle DNA extractions, we used 3-5 beetles per extraction and removed the elytra and wings from individual beetles. DNA extraction was performed using an Xploregen kit, and the integrity and purity of the DNA were evaluated through 1.2% agarose gel electrophoresis.

2.2 Sequencing and analysis of V3-V4 region of 16s rRNA gene

DNA extraction from alcohol-free samples was performed using QIAamp DNA extraction Kit (Qiagen, Germany) according to the standard protocol of genomic DNA extraction. RNA contamination in the specimens was eliminated by adding 2 µl of RNase A (10 mg/ml; Fermentas, USA) to each 20 µl of DNA dissolved in Tris-EDTA buffer (pH 8.0), as well as by incubating at 37°C for 3-4 h. The 16s rRNA gene was amplified using the universal primers V34F-5'AGAGTTTGATGMTGGCTCAG3' and V34R-5' TTACCGCGGCMGCSGGCAC3'. The thermal cycling conditions were an initial denaturation at 95°C, 25 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 2 minutes. A final extension was done at 72°C for 10 minutes, with a hold at 4°C. The quality of the PCR product was evaluated using 1.2% agarose gel electrophoresis. The DNA of a male as well as a female beetle was selected for microbiome analysis. For each sex, a specimen of the pooled DNA of male and female beetles were prepared for sequencing. The amplicons from each sample were purified with ampure beads to eliminate unused primers, and an additional 8 cycles of PCR were conducted with Illumina barcoded adapter to create the sequencing libraries. The libraries were then purified with Ampure beads and quantified using the Qubit dsDNA high sensitivity assay kit. Sequencing was performed using the Illumina Miseq with the 2x300PE v3-v4 sequencing kit.

2.3 Bioinformatics analysis

The analysis of the 16s V3-V4 region was conducted using the NCBI database. The sequencer generates bcl data, which was subsequently de-multiplexed into fastq raw data. To verify the quality of the de-multiplexed data, Fastqc (Version

0.11.9) and Multiqc (Version 1.10.1) tools were employed for quality assessment. Once the sequencing run was finished, a final raw OTU (Operational Taxonomic Unit) table was created, which acts as the foundation for visualizing the analysis. The top 15 organisms in each sample were determined and abundance feature tables were created using Microsoft Excel. Additional analyses were conducted using Microbiomeanalyst (accessible online at https://www.microbiomeanalyst.ca/), including Rarefaction curve, Heatmap, Alpha diversity, PCOA plot, and another online tool metagenessist (http://www.metagenassist.ca/) was used to visualized metabolic pathways.

3. RESULTS

3.1 Alpha diversity

his metagenomics research examined the genomes of both male and female *Z. bicolorata*. The female genome showed a GC content of 54.5%, with a total of 2.2 million reads collected, while the male genome also had a GC content of 54.5%, but only 0.4 million reads were available for analysis. These results offer important insights into the genetic makeup of both sexes of *Z. bicolorata*. The microbial diversity in the samples was assessed using Chao1, Simpson, Fisher, and Shannon metrics, along with observed species metrics, with the findings illustrated in Figure 1. The Chao metric results indicate that female *Z. bicolorata* possesses greater species richness than their male counterparts, as shown in Figure 1(a). In Simpson and Shannon metrics, the male beetle harbors higher species diversity as compared to the female beetle Figure 1 (b, c). Similarly, fisher indices show a higher number of species found in the male beetle Figure 1(d).

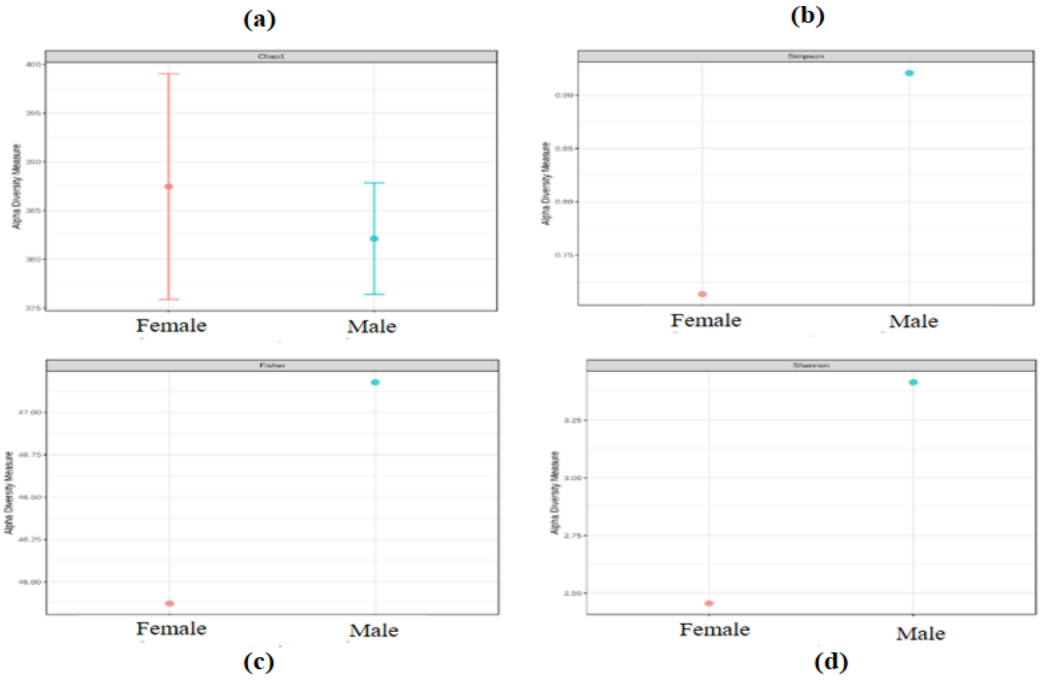


Fig. 1: Metrices: **(a)** Chao1, **(b)** Simpson, **(c)** Fisher and **(d)** Shannon showing species diversity, richness and count of unique OTUs identified from the Male and Female *Zygogramma bicolorata*.

3.2 Microbial diversity

An investigation was conducted to examine the bacterial communities linked to *Z. bicolorata*. The metagenomic DNA from *Z. bicolorata* was extracted using the 16 S rRNA sequencing method [74]. The Illumina Next Generation Sequencing platform was utilized to identify the complete bacterial population present in the beetle. The most prevalent phylum found in males was Proteobacteria, accounting for 67.17%, followed by Firmicutes at 15.26%, Cyanobacteria at 6.39%, Bacteroidetes at 6.30%, and Actinobacteria at 4.14%. All other phyla had an abundance of less than 1%. Similarly, in the case of female *Z. bicolorata*, Firmicutes was found to be abundance phyla which constitute about (57.53%) followed by proteobacteria (28.05%), Actinobacteria (8.4%), Cyanobacteria (2.6%) and other constitute abundance of less than 1% (Figure 2a and 2b; Figure 11 and 12). Whereas in class level Gammaproteobacteria was dominant (42.4%) followed by the Betaproteobacteria (20%), Bacilli (9.7%), Un-classifying Cyanobacteria (6.3%) and other constitutes in lower numbers in the case of a male. Comparatively, in female beetle Bacilli was found to be the most dominant Bacilli (55.19 %) followed by the Gammaproteobacteria (20.4%), Actinobacteria (8.3%), Betaproteobacteria (3.5%) were most abundant (Figure 3 a and b). Similarly, the enterobacterales (21.3%) which was predominant in male beetle followed by Pseudomonales (19.2%) and Burkholderiales (18.4%) likewise Bacillales (53.7%) and enterobacterales (11%) and pseudomonadales (7.8%) in female (Figure 4 a and b). Enterobacteriaceae (19.6%) and Comamonadaceae (16.6%) bacterial family showed richness in male beetle whereas Bacillaceae (52.8%) followed by the Enterobacteriaceae (9.3%) and Streptomycetaceae (7%) in female beetle (Figure 5 a and b).

In the same way, Candidatus (15.6%), Pseudomonas (14.9%), Escherichia (13.9%), Synechoccus (6.3%) etc. were the predominant genera in male beetles meanwhile Bacillus (52%), Streptomyces (7%), Escherichia (5.3%) Pseudomonas (4.8%) etc were the most in female as shown in (Figure 6a and 6b). The species found in male beetle *are Candidatus symbiobacter mobilis* (15.6%), *E. coli* (13.9%), Pseudomonas yamanorum (8.7%), etc. while in female *Bacillus velezensis* (40.5%) which was most dominant species followed by *Bacillus amyloliquefaciens* (6.9%), *Streptomyces sp.* (6.8%), *E. coli* (5.3%) and *Bacillus Sp.* (3.3%) was found to be abundant (Figure 7a and 7b).

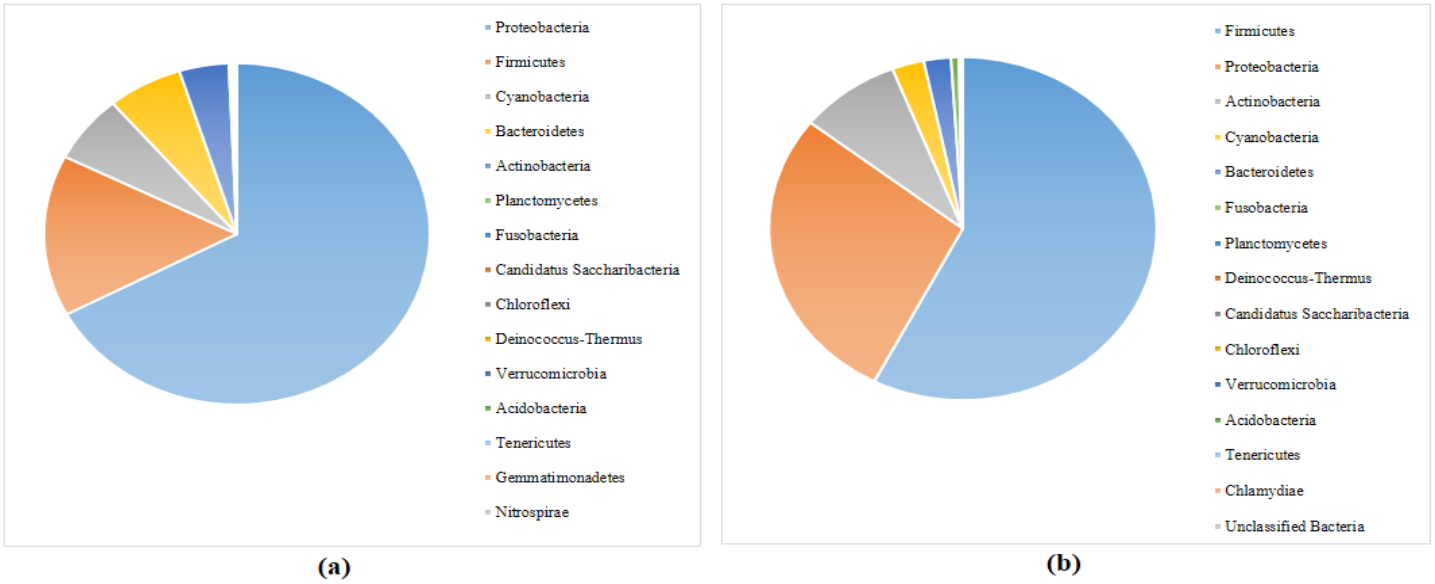


Fig 2: Showing abundance of bacteria at phylum level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.

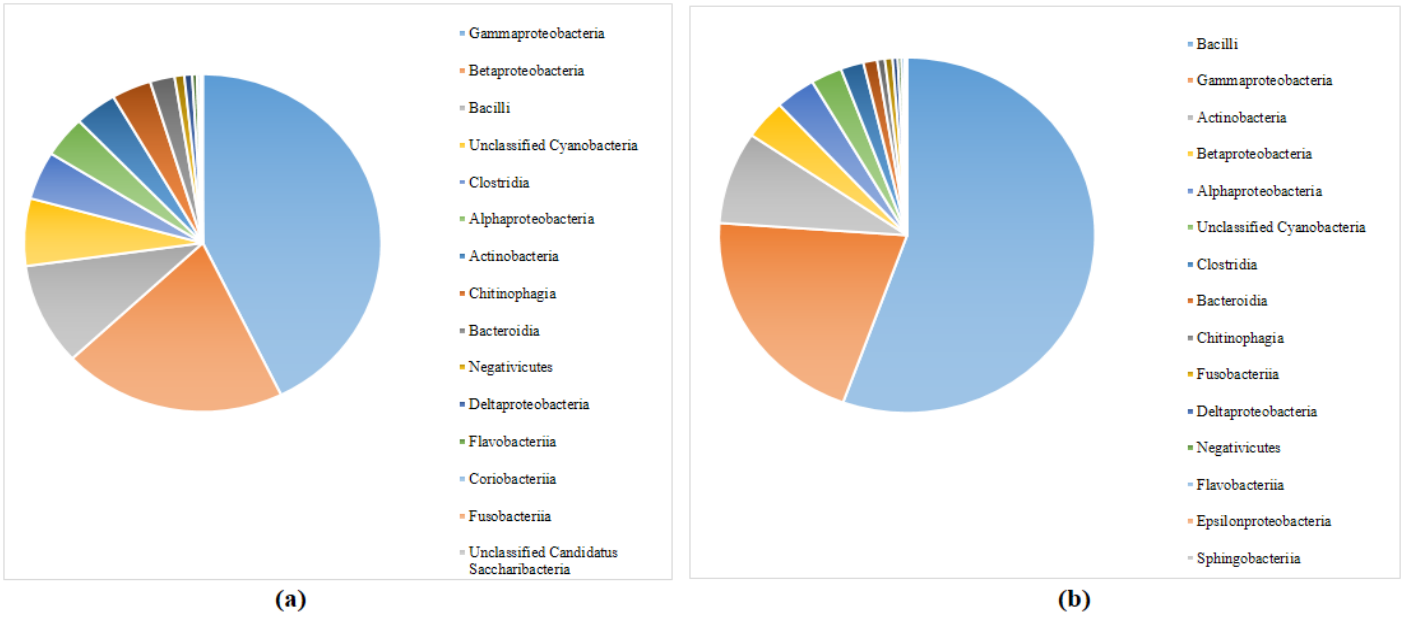
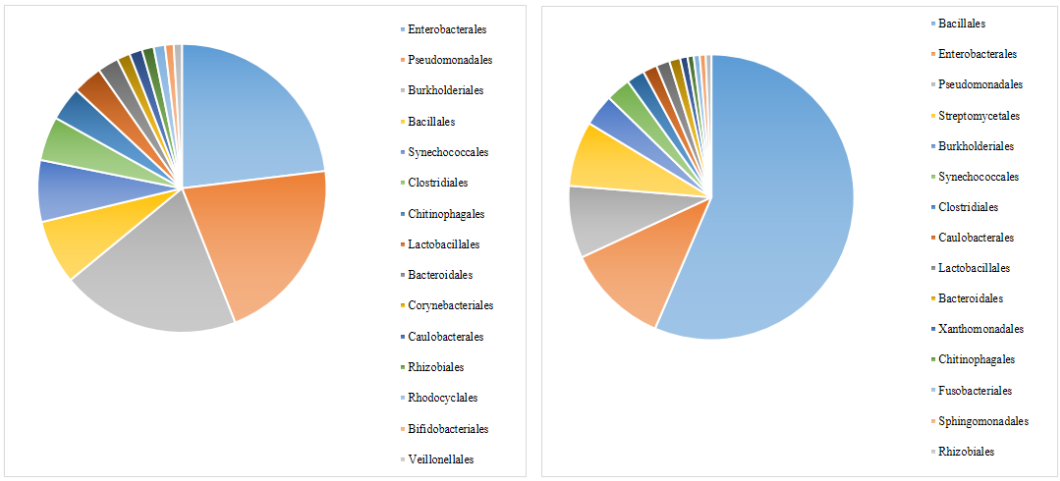
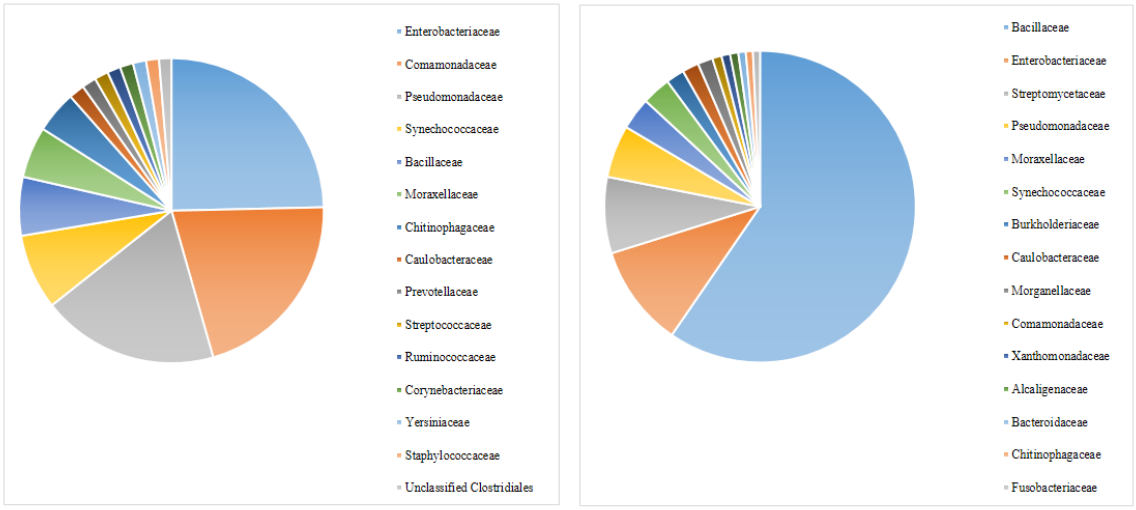


Fig 3: Showing abundance of bacteria at Class level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.



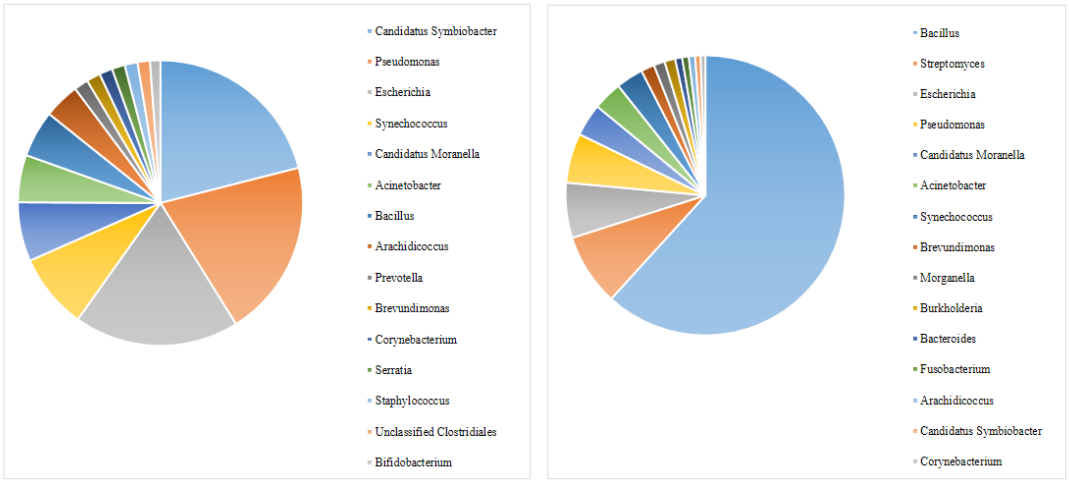
(a) **(b)**

Fig 4: Abundance of bacteria at order level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.



(a) **(b)**

Fig 5: Showing abundance of bacteria at Family level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.



(a) **(b)**

Fig 6: Showing abundance of bacteria at Genus level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.

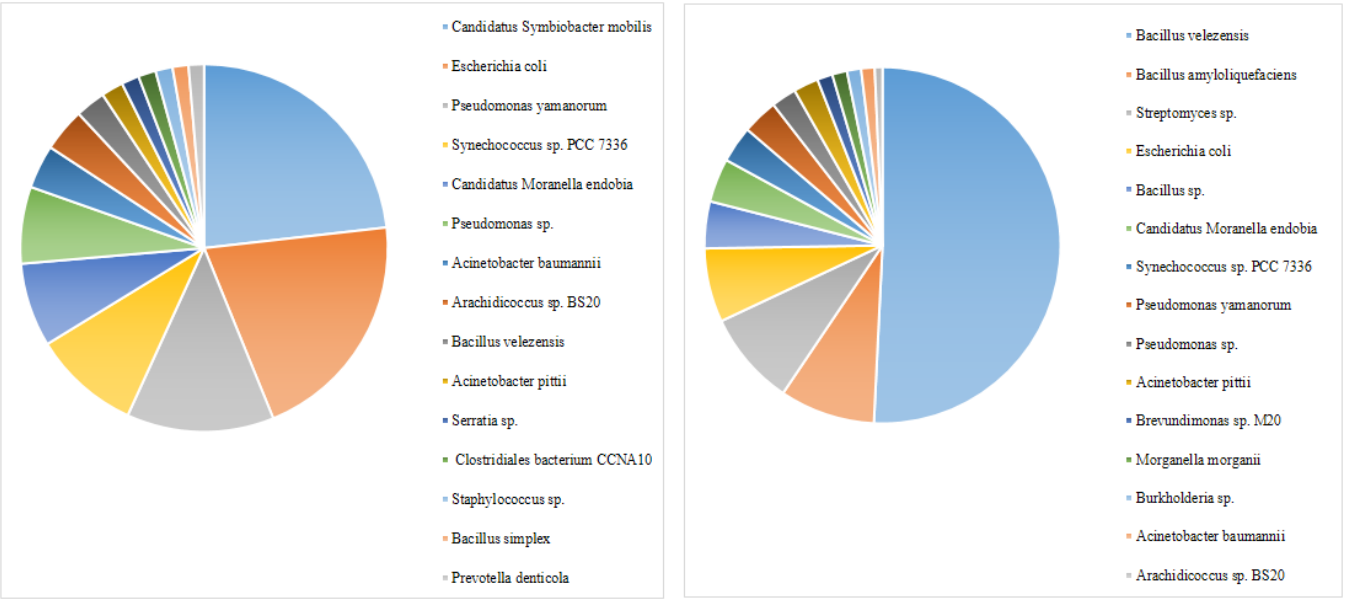


Fig 7: Showing abundance of bacteria at Species level in: **(a)** Male and **(b)** Female of *Z. bicolorata*.

4. DISCUSSION

This study provides the first comprehensive analysis of the bacterial microbiota associated with both male and female *Z. bicolorata*, elucidating critical relationships between gut microbial composition, dietary ecology, and the beetle's functional role as a biological control agent. The findings demonstrate that *Z. bicolorata* harbors diverse microbial communities that appear to contribute to host nutrition, physiology, and ecological adaptability.

Prokaryotes have co-evolved with diverse microbial consortia that colonize the internal and external surfaces of insects, forming complex and dynamic symbiotic systems [75]. These associations often influence host fitness more profoundly through community-level interactions than through individual taxa alone. Insects acquire these microbial partners either vertically, from parent to offspring, or horizontally, via environmental exposure or diet [76]. Such symbioses are well known to play essential roles in nutrient digestion, cellulose degradation, nitrogen fixation, detoxification, and the biosynthesis of essential metabolites, thereby enhancing the host's metabolic efficiency and ecological success [77]. Our analysis revealed that the bacterial microbiota of *Z. bicolorata* is dominated by members of the phyla Firmicutes and Proteobacteria (Figure. 2a and 2b). In males, Proteobacteria accounted for the largest proportion (67.17%), followed by Firmicutes (15.26%), Cyanobacteria (6.39%), Bacteroidetes (6.30%), and Actinobacteria (4.14%), while other phyla were present at <1%. In females, Firmicutes predominated (57.53%), followed by Proteobacteria (28.05%), Actinobacteria (8.4%), and Cyanobacteria (2.6%), with minor contributions from other phyla. These patterns are consistent with prior studies in *Samia ricini* [78-79], where Proteobacteria and Firmicutes were similarly dominant, accounting for ~60% and ~20% of the gut bacterial communities, respectively.

The dominance of these two phyla aligns with reports from other phytophagous beetles, including stem-feeding *Rhynchophorus ferrugineus*; [80], wood-feeding *Dendroctonus spp*.; [81], and rice-feeding *Lissorhoptrus oryzophilus*; species [82]. Comparable microbial assemblages have also been reported in termites, where Firmicutes, Bacteroidetes, and Spirochaetes are integral to cellulose degradation and nutrient cycling [83]. Collectively, these microbial groups, particularly Proteobacteria, Firmicutes, Clostridia, and Actinomycetes have been implicated in modulating host development, digestion, immunity, and ecological adaptation [84-86].

Interestingly, marked sex-specific differences were observed in the bacterial community structures of *Z. bicolorata*. In males, dominant genera included Candidatus, Symbiobacter (15.6%), Pseudomonas (14.9%), *Escherichia* (13.9%), and Synechococcus (6.3%). In contrast, female beetles harbored higher relative abundances of Bacillus (52%), Streptomyces

(7%), *Escherichia* (5.3%), and Pseudomonas (4.8%) (Figs. 6 a, b, 8). These differences suggest that sexual dimorphism in physiology and metabolism may shape the composition and functional potential of symbiotic bacterial communities [87].

Previous studies have shown that certain Pseudomonas species found in *Leptinotarsa decemlineata* oral secretions can suppress host plant defenses [88-89]. Similarly, the gut microbiota of *Dendroctonus ponderosae* includes Serratia, Pseudomonas, and Rahnella, which harbor genes essential for terpene degradation, aiding in host detoxification [90-91]. In *Z. bicolorata*, males exhibited higher proportions of Candidatus, Symbiobacter and Pseudomonas, while females were enriched in Bacillus and Streptomyces, suggesting sex-specific metabolic contributions. Candidatus species, such as those found in whiteflies, provide essential amino acids and carotenoids not synthesized by the host [92, 93]. Likewise, *Streptomyces spp*., known for their strong metabolic versatility, may aid nutrient acquisition, while *Pseudomonas* species exhibit enzymatic activities (cellulolytic, xylanolytic, lipolytic) that enhance digestion [94, 95]. The presence of *Escherichia coli*, which may form mutualistic relationships with *Pantoea stali*, further supports the hypothesis that gut bacteria contribute to amino acid and vitamin biosynthesis, as observed in other hemipterans (Nikoh [96-97]. Sex-based microbial differences may also result from distinct immune responses and metabolic demands [98-99], as well as behavioral and physiological factors (Fig. 9, 10).

Overall, this study constitutes the first metagenomic characterization of the bacterial microbiota associated with *Z. bicolorata*. The results indicate that the beetle's gut community is predominantly composed of environmentally derived symbiotic bacteria, with a relatively low representation of pathogenic taxa. Interestingly, male-killing bacteria, including *Spiroplasma*, *Wolbachia*, and *Rickettsia spp*., were detected in both sexes, albeit with variable abundances. These findings not only expand the understanding of *Z. bicolorata*'s symbiotic ecology but also highlight the potential influence of sex-specific microbial assemblages on the species' success as a biological control agent.

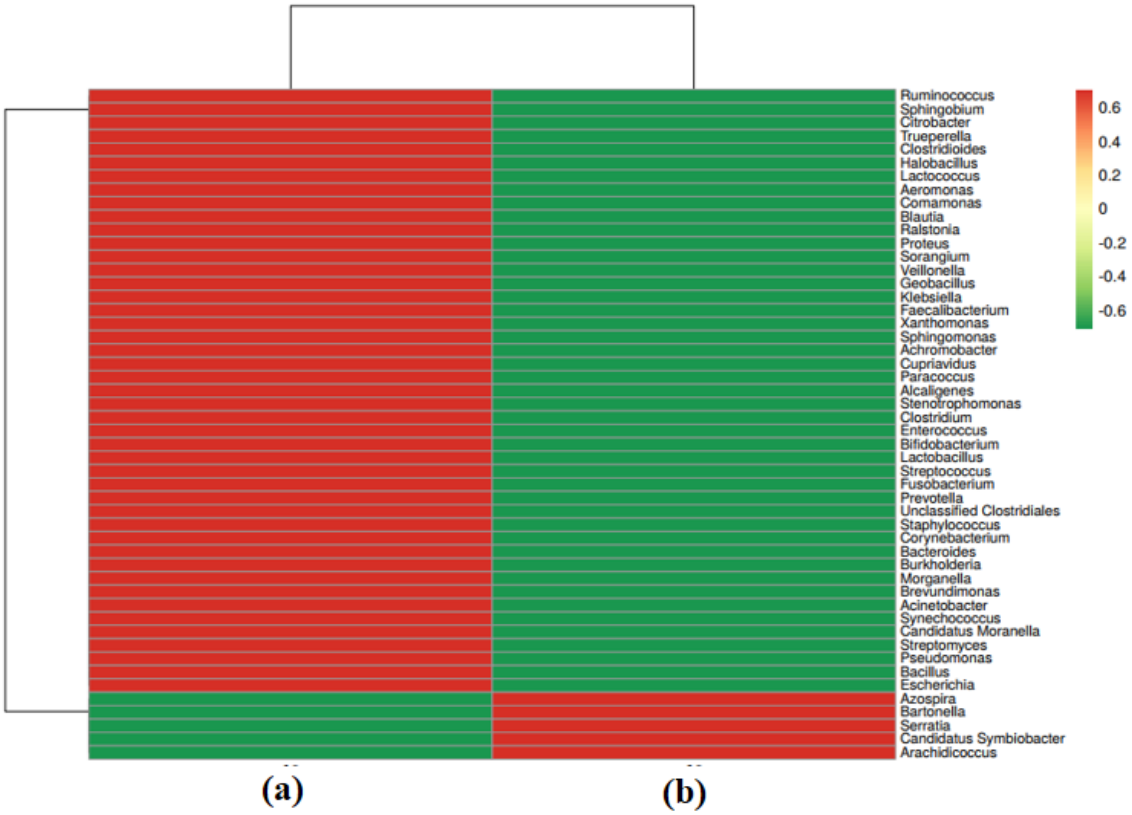


Fig 8: Heatmap of representative sequences of operational taxonomic units (OTUs) in: **(a)** Female and **(b)** Male of *Z. bicolorata*. The range of colours indicates the OTUs relative abundance for each sample. Red colours indicate higher abundance and Green ones less abundances

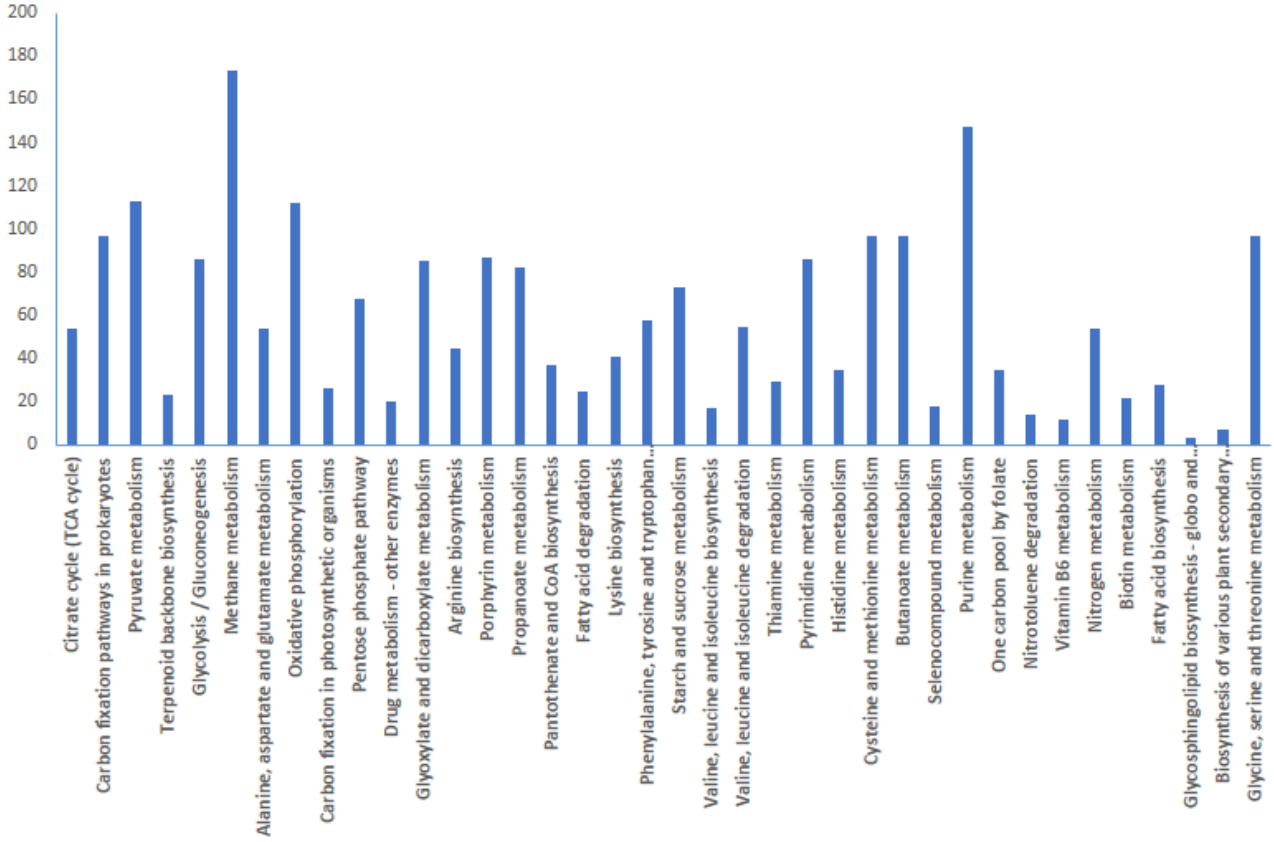


Fig 9: The diversity of bacteria in male beetle take part in functional metabolism

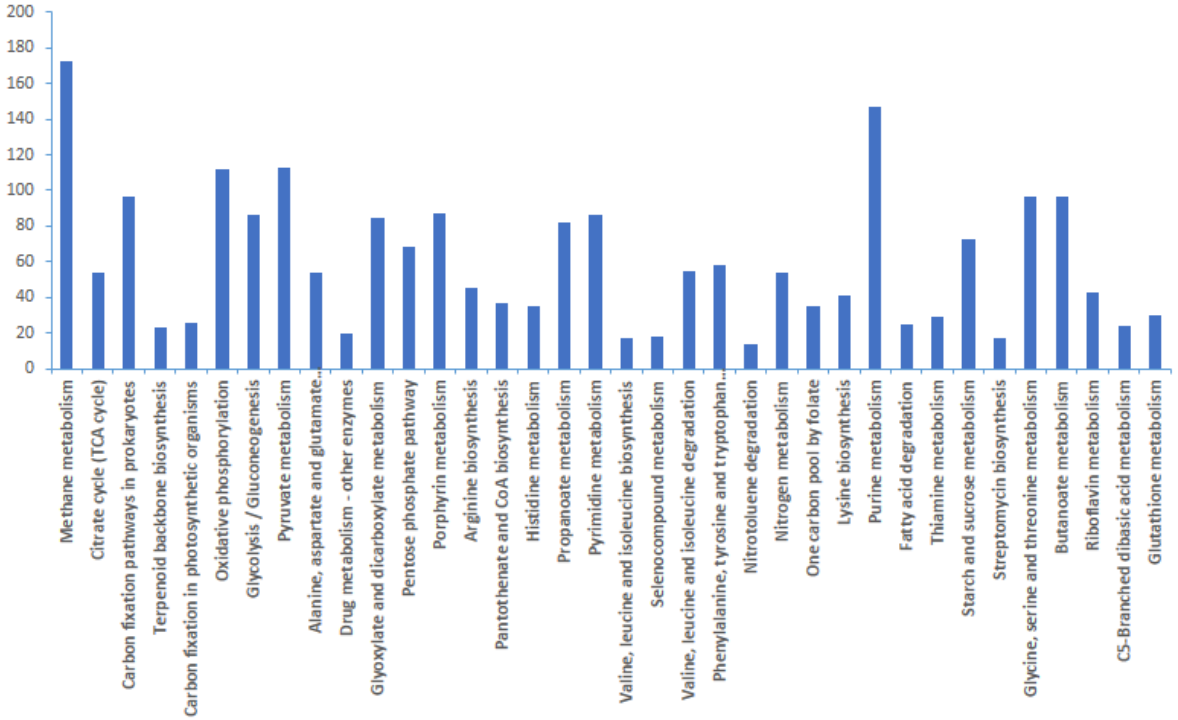


Fig 10: The diversity of bacteria in female beetle take part in functional metabolism

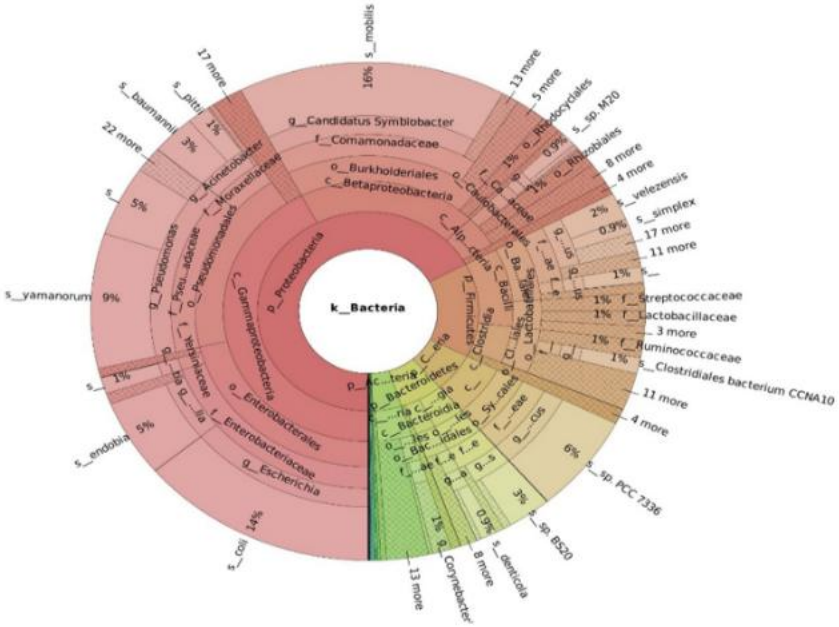


Fig 11: Krona chart of the bacteria represented by 16S rRNA gene amplicon-based bacterial diversity in a male *Z. bicolorata*. Each circle represents the phylum, class, order, family, genus, and species from the inside to the outside of the circle, respectively, indicated by percent diversity based on the absolute number of representative bacteria

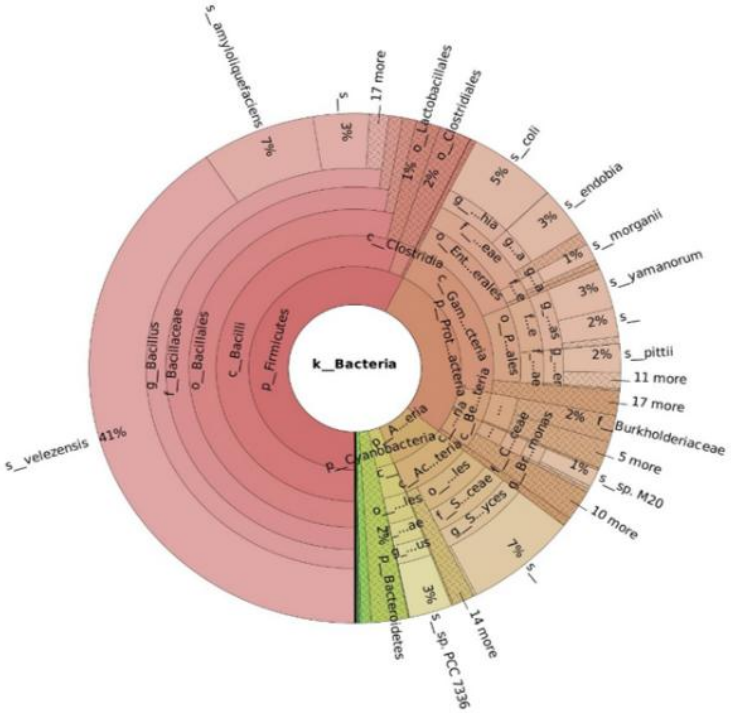


Fig 12: Krona chart of the bacteria represented by 16S rRNA gene amplicon-based bacterial diversity in a female *Z. bicolorata*. Each circle represents the phylum, class, order, family, genus, and species from the inside to the outside of the circle, respectively, indicated by percent diversity based on the absolute number of representative bacteria

5. CONCLUSIONS

This study highlights significant associations between the bacterial communities in both the sexes of *Z. bicolorata* and their ecological as well as nutritional functions. Through 16S rRNA metagenomic analysis, we characterized the bacterial diversity and explored sex-based differences in microbial composition. The results revealed that a majority of the bacterial taxa were unculturable and that the internal microbiota was both diverse and abundant. Moreover, the bacterial assemblages differed between sexes, with males exhibiting greater microbial diversity than females. These findings advance our understanding of the microbial ecology of *Z. bicolorata*, provide insights into its reproductive strategies, and establish a foundation for future research on the beetle's interactions with symbiotic microorganisms and their potential applications in biological control programs.

Ethics Declaration: Not Applicable

Funding Declaration: Not available for this study

Conflict of Interest All the authors declare that they have no conflicts of interest.

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Author Contributions
Khomesh Hiwraj Lanjewar Conduct of experiment, Data curation, writing - original draft, visualization, investigation. Lankesh Yashwant Bhaisare helped in visualization, drafting and editing of the manuscript. Sandeep Kaushik helped in methodology, software, validation, writing - review and editing. Desh Deepak Chaudhary conceptualization, methodology, software, validation, supervision, writing - review and editing.

Data Availability: All data involved in this study are available upon reasonable request made to the corresponding author.

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