TROPICAL LIFE SCIENCES RESEARCH

Characterisation of 16S rRNA Metagenomic Amplicon Data of Bacterial Communities Associated with IGR Treated Tropical Bed Bugs, *Cimex hemipterus* (F.) (Hemiptera)

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HIGHLIGHTS

- Metagenomic characterisation of bacterial communities in insecticide-treated *Cimex hemipterus*, revealing Proteobacteria (> 99%) as the dominant phylum across all samples.
- Wolbachia and Burkholderia-Caballeronia-Paraburkholderia were identified as key endosymbionts, with pyriproxyfen treatment showing distinct shifts in their abundance compared to control samples.
- Insecticide exposure has minimal impact on overall gut bacterial diversity, but subtle changes in secondary symbionts suggest potential symbiont-mediated mechanisms influencing insecticide susceptibility.

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Characterisation of 16S rRNA Metagenomic Amplicon Data of Bacterial Communities Associated with IGR Treated Tropical Bed Bugs, *Cimex hemipterus* (F.) (Hemiptera)

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Abstract: Endosymbionts serve crucial physiological functions within their insect hosts, including nutrition provision, metabolic enhancement and detoxification stimulation. The exploration of the microbiome population makeup corresponding to tropical bed bugs (*Cimex hemipterus*) became feasible with the advancement of novel metagenomic approaches. This study examined the microbial composition of tropical bed bugs treated with four different classes of insecticides: chlorfluazuron, tebufenozide, pyriproxyfen, β-cyfluthrin + imidacloprid and control. Next, an Illumina MiSeq was used to sequence the hypervariable region (v3–v4) of the 16S rRNA (ribosomal RNA) gene. Proteobacteria accounted for over 99% of the overall microbial population in all samples. The two most common operational taxonomic units (OTUs) at the genus level were Wolbachia, an alpha-proteobacterium and Pectobacterium, a gamma-proteobacterium. Despite some similarities in the major OTUs of the bacteria, the genera represented in the lower abundances were quite diverse. The bacterial abundance of bed bugs treated with pyriproxyfen exhibited a notable disparity from the control sample. This research lays the groundwork for a previously unknown symbiont-mediated mechanism influencing insecticide susceptibility in bed bugs.

Keywords: Endosymbionts, *Cimex hemipterus*, Metagenomic, Proteobacteria

Abstrak: *Endosimbion memainkan peranan penting dalam fisiologi serangga perumah, termasuk biosintesis nutrien, modulasi metabolisme dan penyahtoksikan. Kemajuan teknologi metagenomik membolehkan pencirian terperinci komuniti mikrobiom, termasuk dalam pepijat tropika Cimex hemipterus. Kajian ini menilai struktur komuniti bakteria dalam C. hemipterus selepas pendedahan kepada empat kelas racun serangga: chlorfluazuron, tebufenozide, pyriproxyfen dan campuran β-cyfluthrin + imidacloprid, dan membandingkannya dengan kumpulan kawalan. Penjujukan kawasan hiperubah V3–V4 gen 16S rRNA menggunakan pendekatan kultur-bebas metagenomik dijalankan untuk mengenal pasti kepelbagaian taksonomi bakteria. Hasil menunjukkan filum Proteobacteria mendominasi (>99%) dalam semua sampel. Pada peringkat genus, Wolbachia dan Burkholderia–Caballeronia–Paraburkholderia merupakan taksa dominan merentas kumpulan rawatan dan kawalan. Indeks kepelbagaian alfa (Shannon, Simpson,*

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Chao1) tidak menunjukkan perbezaan signifikan (p > 0.05). Walaupun demikian, beberapa OTU memperlihatkan perubahan kelimpahan relatif, terutama pada sampel yang dirawat dengan pyriproxyfen. Keseluruhannya, pendedahan kepada racun serangga tidak menjejaskan kepelbagaian bakteria secara menyeluruh, namun perubahan selektif pada endosimbion tertentu mencadangkan interaksi antara mikrobiom dan respons fisiologi terhadap tekanan kimia. Dapatan ini menyediakan asas untuk penyelidikan lanjut mengenai peranan simbiosis mikrob dalam mekanisme toleransi atau kerentanan serangga terhadap racun serangga.

Kata kunci: *Endosimbion, Cimex hemipterus, Metagenomik, Proteobacteri*

INTRODUCTION

Cimex is a well-known hematophagous species, with *Cimex lectularius* and *Cimex hemipterus* being the most closely related to humans. Bed bugs were initially described as the prey of bats, birds and mammals, but they successfully adapted to humans who shared the same cave in the Mediterranean and Central Asia regions (Akhoundi *et al.*, 2020). As urbanisation and civilisation advance, humankind makes an effort to enhance its living conditions, and bed bugs adapt to this new way of life in tandem. As civilisation and urbanisation progress, humans begin to strive to improve their living conditions and bed bugs evolve alongside them to the new lifestyle. Similar to mosquitoes, a blood meal serves as the essential nutritional source required for the survival, development and reproduction of this cimicid species (Akhoundi *et al.* 2020; Dieng *et al.* 2017). Hence, they skilfully locate their host by the emanation of body odour, heat and carbon dioxide from their sleeping hosts (Doggett *et al.* 2012; Goddard & DeShazo 2009). Bed bugs, belonging to the hemimetabolous insect order, progress through seven life stages, where each stage needs a blood meal for development. The entire life cycle of bed bugs, from oviposition to pupation, typically spans between 30 and 70 days, yet this duration is highly contingent upon environmental conditions and the availability of hosts (Harlan 2006; Delaunay *et al.* 2011; Okwa & Omoniyi 2016). Interestingly, this robust pest can survive months without blood meals, besides their fast population advancement, making them difficult to combat. It was widely documented in history that mankind had successfully overcome the burden of this formidable ectoparasite, yet recently it had spread over the world like a plague, especially in developed areas like Europe, North America, Australia and Eastern Asia (Doggett *et al*. 2004; Potter 2007). The present rise was prompted by numerous factors, including travel, global warming, recycling infested furniture, periodic usage of bait traps with chemical residue and establishment of resistance across the bed bug population (Benoit 2007; Lim & Majid 2021). The resistance mechanism plays a pivotal role in the re-infestation issue, where bed bug capable of modulating three types of resistance competence, including behavioural resistance, physiological resistance

and symbiont-mediated resistance. According to Rupawate *et al.* (2023), symbionts often fulfill the nutritional requirements of their hosts, support general growth, aid in reproduction, protect against pathogen infections, control host gene expression and undertake biochemical detoxification of toxins and pesticides. This phenomenon allows bed bugs to persist and extend their life cycle in spite of being subjected to a range of treatment methods. Bedbugs are hosts to a diverse array of microbial symbionts, which exist in varying quantities. Among these, Wolbachia is widely acknowledged as one of the most ubiquitous microorganisms found in bedbugs, because it provides their host insect with nutrients such as vitamin B (Lim & Majid 2021). 16S rRNA metagenomics analysis is widely employed for exploration of microbiota of a sample host, as this new-generation sequencing analysis amplifies the V3-V4 hypervariable region to identify the bacterial diversity (Bukin *et al.* 2019; Gupta *et al.* 2019). To further understand the impacts of insecticides on the bacterial population of *C. hemipterus*, samples were examined and characterised employing a 16S rRNA (Ribosomal RNA) metagenomic culture-independent technique, subsequently followed by MiSeq Illumina sequencing.

MATERIAL AND METHOD

Bed Bug

Tropical bed bugs were collected from residential houses around Penang Island and mass-reared in a laboratory setting to imitate their habitats to promote their longevity and reproduction. Employing surface contact bioassay, five adults per replicate were treated with four classes of insecticides at four doses for a 2-week interval. Then, the *C. hemipterus* were preserved in 95% ethanol for 16S rRNA metagenomic culture-independent analysis.

Microbial DNA Extraction

A single bed bugs from each replicate of bioassay treatment and control were selected and sterilised using 70% ethanol and then drowned in distilled water for molecular analysis (Cappelli *et al.* 2022). HiYield Genomic DNA isolation kit (Real Biotech Corporation, Taiwan) was used to separate microbial DNA from the bed bug sample. The concentration and purity of the microbial DNA samples extracted were evaluated using a Quawell Micro Volume Spectrophotometer (Labgene Scientific, Switzerland) and visualised on agarose gels.

16S Illumina Library and Sequencing

Samples of microbial DNA that had been extracted were delivered to a service provider to undergo library assembly and 16S rRNA metagenomic identification. 20 microliters mixture of 5 FastPfu Buffer (4 µL), 2.5 mM dNTPs (2 µL), primer 5 µM (0.8 µL), FastPfu Polymerase (0.4 µL) and template DNA (10 ng) were utilised. The polymerase chain reaction method was used to make copies of the bacteria's marker region. The procedure included 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The primer sequence used was 341F-80R.

16S Metagenomic Data Analysis

The raw FASTQ files were demultiplexed and quality checked with QIIME (version 1.9.1). The readings were utilised for community diversity assessment, taxonomy classification and OUT clustering. A standard sequence number corresponding to the sample with the fewest sequences was used to normalise the OTU abundance data. At a similarity threshold of 97%, operational taxonomic units (OTUs) were selected and identified; OTUs lacking a bacterial phylum assignment or originating from chloroplasts or mitochondria were omitted. The β-diversity revealed the richness and evenness of each sample's bacterial community. Paired T-tests were used to compare bacterial genus abundance in insecticide-treated and untreated samples.

RESULTS

Data Summary

On average, there are 49,417,842 reads in the *Cimex hemipterus* control sample. In contrast, the treated tropical bed bugs display marginally higher numbers of reads when exposed to pyriproxyfen (50,392,421), tebufenozide (50,525,885), □-cyfluthrin + imidacloprid (49,463,607) and chlorfluazuron (42,414,568). A higher species richness was revealed by the identification of a greater number of OTUs in treated bed bugs (Table 1). The data are accessible via NCBI SRA with the identification number: PRJNA918835 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA918835/)

TABLE 1. Data summary for each bed bug sample.

Sample/Replicate	Number of reads (bp)	Average length (bp)
Control (C1)	54,664,740	409.44
Control (C2)	44,170,945	405.54
Chlorfluazuron (CH1)	43,279,718	406.15
Chlorfluazuron (CH2)	41,549,419	408.24
Pyriproxyfen (P1)	44,158,666	426.17
Pyriproxyfen (P2)	56,626,176	424.94
Tebufenozide (T1)	46,267,932	407.44
Tebufenozide (T2)	54,783,838	409.06
β-Cyfluthrin + Imidacloprid (TM1)	50,909,020	406.18
β-Cyfluthrin + Imidacloprid (TM2)	48,018,195	405.75

The Shannon and Simpson indices both point to a reduced species richness in treated samples compared to untreated samples. On the other hand, for every sample, the Chao1 index predicted higher species richness. Overall, there is some variation in the bacterial assemblage richness across the bed bug samples. However, when comparing the sequences using various alpha diversity metrics such as Shannon, Simpson and CHAO1, there were no significant differences (Shannon, $p = 0.254$; Simpson, $p = 0.321$; CHAO1, $p = 0.183$) between the control and treated bed bug groups. However, when comparing treated and untreated bed bugs, the rarefaction curves showed that treated bed bugs had steeper and higher curves, indicating a higher taxonomic richness (Fig. 1). There is a possibility that these samples contain additional OTUs that have not been discovered, as indicated by the curves yet to hit a plateau. This could potentially have an impact on the Shannon and Simpson indices.

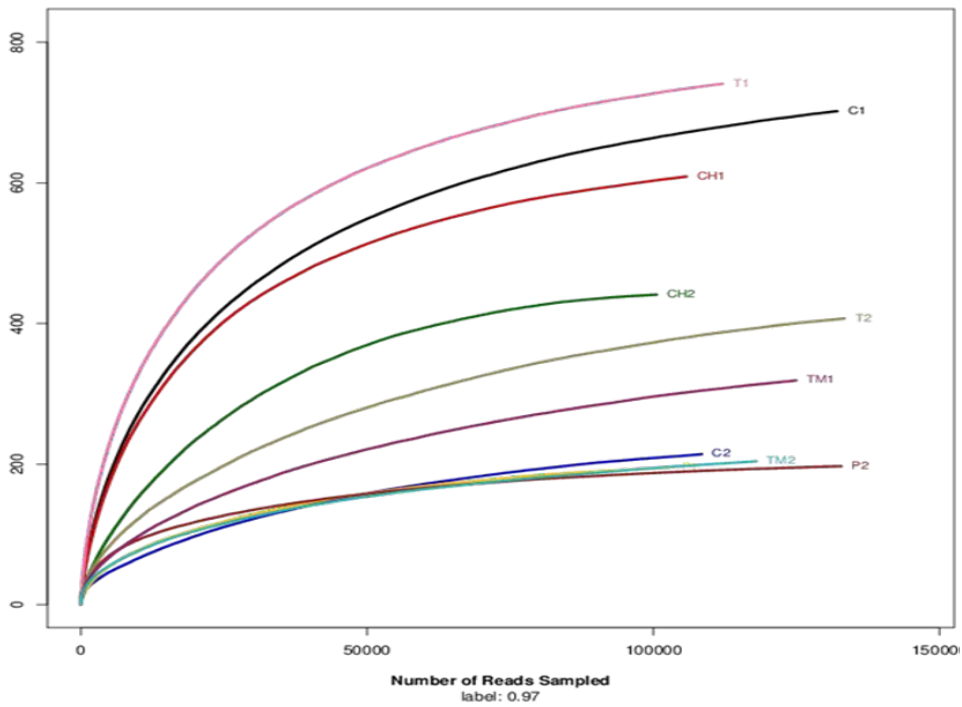

FIGURE 1: Rarefaction curves of control and insecticide-treated tropical bed bug samples.

Notes: C1 = Control 1; C2 = Control 2; CH1 = Chlorfluazuron 1; CH2 = Chlorfluazuron 2; T1 = Tebufenozide 1; T2 = Tebufenozide 2; TM1 = β-cyfluthrin + Imidacloprid 1, TM2 β-cyfluthrin +Imidacloprid 2; P1 = Pyriproxyfen 1; P2 = Pyriproxyfen 2.

Microbial Community

As shown in Fig. 2, the heatmap indicated that Proteobacteria constituted the predominant phylum, exhibiting relative abundances exceeding 99% in all samples. Less than 1% of the remaining OTUs were classified as belonging to the phyla Actinobacteria, Firmicutes, Fibrobacterota, Spirochaetota and others (Fig. 3). The bacterial genera/species shared between all samples are shown in Table 2.

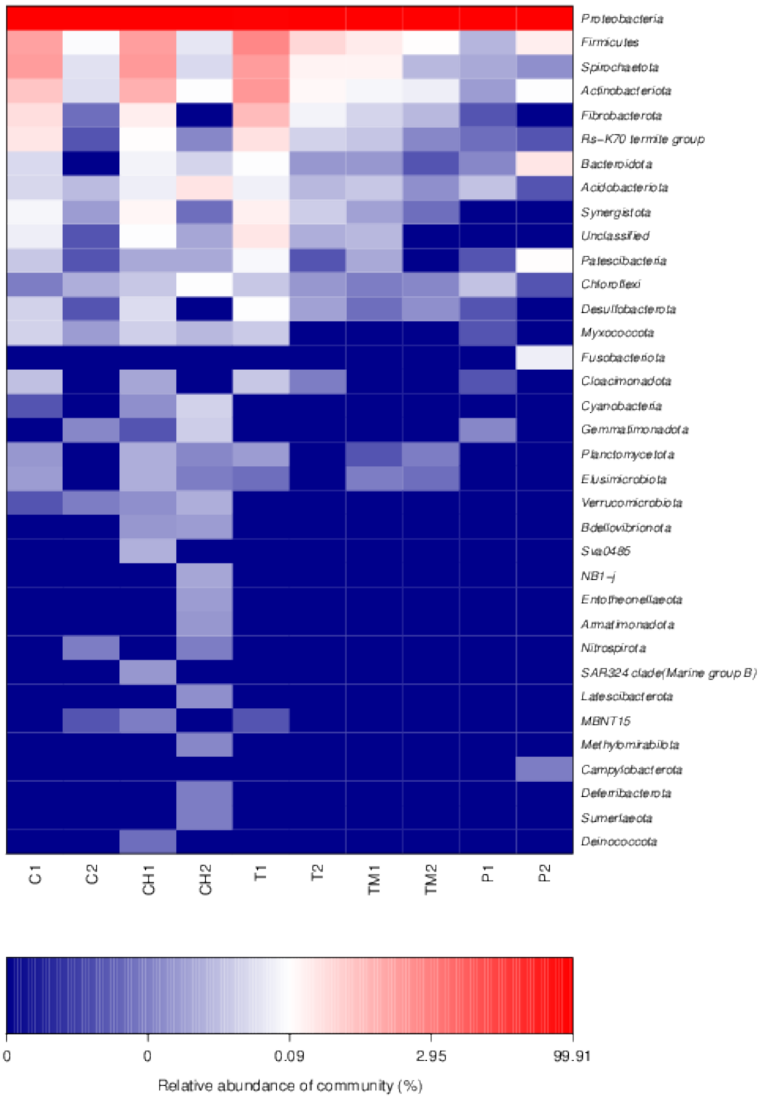

FIGURE 2: Heatmap analysis of 10 samples, C1, C2, C1, C2, T1, T2, TM1, TM2, P1 and P2.

Notes: C1 = Control 1; C2 = Control 2; CH1 = Chlorfluazuron 1; CH2 = Chlorfluazuron 2; T1 = Tebufenozide 1; T2 = Tebufenozide 2; TM1 = β-cyfluthrin +Imidacloprid 1, TM2 β-cyfluthrin + Imidacloprid 2; P1 = Pyriproxyfen 1; P2 = Pyriproxyfen 2.

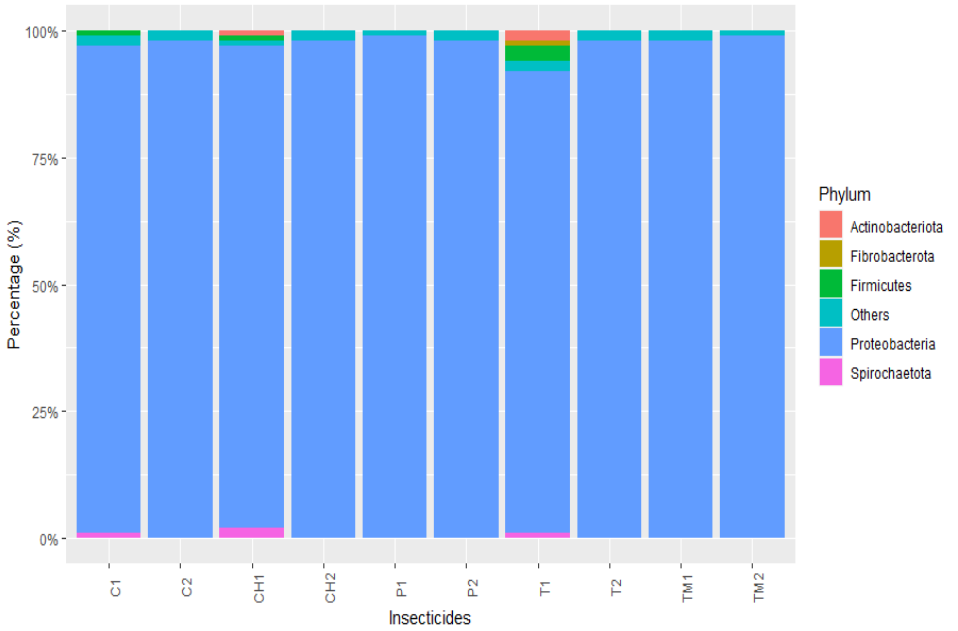

FIGURE 3: Summary of bacterial phyla (OTUs) of insecticides treated (CH1, CH2, P1, P2, T1, T2, TM1, TM2) and untreated (C1, C2) *Cimex hemipterus*.

Notes: C1 = Control 1; C2 = Control 2; CH1 = Chlorfluazuron 1; CH2 = Chlorfluazuron 2; T1 = Tebufenozide 1; T2 = Tebufenozide 2; TM1 = β-cyfluthrin + Imidacloprid 1, TM2 β-cyfluthrin +Imidacloprid 2; P1 = Pyriproxyfen 1; P2 = Pyriproxyfen 2.

The concept of the "relative abundance of identified bacterial taxa" pertains to the ratio of reads corresponding to individual taxa to the sum of all reads for all taxa. Among the bacterial genera, there were five most abundant genera, including *Wolbachia, Burkholderia-Caballeronia-Paraburkholderia, Pectobacterium, Sanguibacter-Flavimobilis* and Termite *Treponema* cluster were present across all samples (Fig. 4). The genus, *Wolbachia* and *Burkholderia-Caballeronia-Paraburkholderia,* greatly monopolised the data sequence. To be precise, *Wolbachi*a were most abundant in the control sample, while *Burkholderia-Caballeronia-Paraburkholderia* were in the pyriproxyfen-treated sample (Table 2).

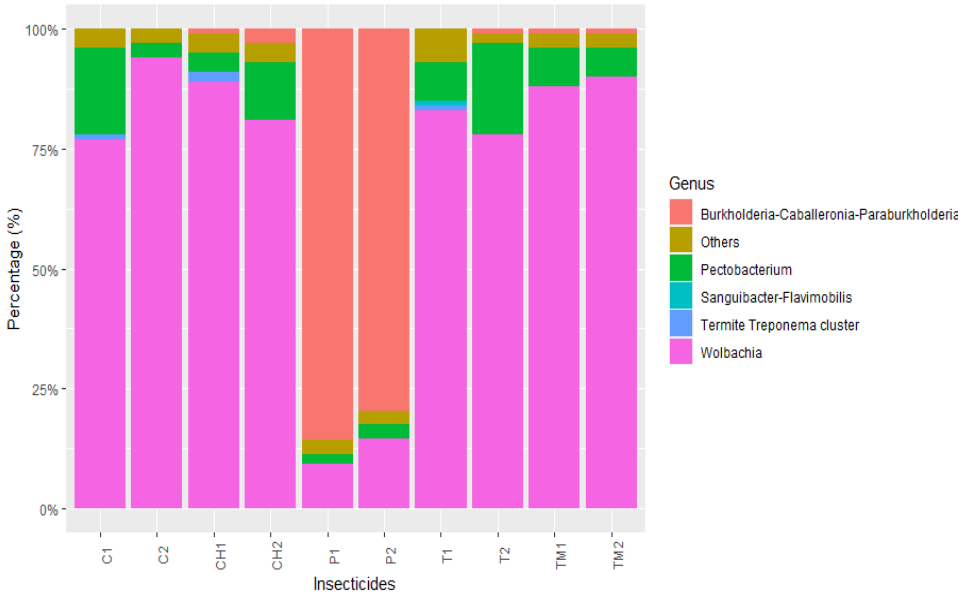

FIGURE 4: Summary of bacterial genera (OTUs) of insecticides treated (CH1, CH2, P1, P2, T1, T2, TM1, TM2) and untreated (C1, C2) *Cimex hemipterus.*

Notes: C1 = Control 1; C2 = Control 2; CH1 = Chlorfluazuron 1; CH2 = Chlorfluazuron 2; T1 = Tebufenozide 1; T2 = Tebufenozide 2; TM1 = β-cyfluthrin +Imidacloprid 1, TM2 β-cyfluthrin + Imidacloprid 2; P1 = Pyriproxyfen 1; P2 = Pyriproxyfen 2.

TABLE 2. Taxonomic classification of bacterial sequences (OTUs) that were shared in all 10 samples of tropical bed bugs.

Taxonomy (Domain; phylum; class; order; family; genus; species)	Number of sequences				
	C1	C2	CH1	CH2	P1
Bacteria; Proteobacteria; Alpha proteobacteria; Rickettsiales; Anaplasmataceae; *Wolbachia; Wolbachia-unclassified*	70,276	49,809	83,166	73,953	10,008
Bacteria; Proteobacteria; Gamma proteobacteria;Burkholderiales; Burkholderiaceae; *Burkholderia-Caballeronia-Paraburkholderia; Burkholderia-Caballeronia-Paraburkholderia-unclassified*	187	3,049	1,296	2,797	86,394
Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; Anaplasmataceae; *Wolbachia; Wolbachia pipientis*	2,353	38,817	1,704	3,940	229

(continued on next page)

Table 2: *(Continued)*

Taxonomy (Domain; phylum; class; order; family; genus; species)	Number of sequences				
	P2	T1	T2	TM1	TM2
Bacteria; Proteobacteria; Alpha proteobacteria; Rickettsiales; Anaplasmataceae; *Wolbachia; Wolbachia-unclassified*	14,374	75,322	70,973	52,509	80,014
Bacteria; Proteobacteria; Gamma proteobacteria; Burkholderiales; Burkholderiaceae; *Burkholderia-Caballeronia-Paraburkholderia; Burkholderia-Caballeronia-Paraburkholderia-unclassified*	78,892	235	654	724	1,133
Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; Anaplasmataceae; *Wolbachia; Wolbachia pipientis*	445	2,653	4,121	33,022	7,645

Paired sample T-test divulges no substantial interactions ($p > 0.005$) in bacterial sequences of insecticide-treated bed bugs when compared against untreated bed bugs. The abundance ratio of the top three most abundant species of bed bugs, *Wolbachia unclassified*, followed by *Burkholderia-Caballeronia-Paraburkholderia unclassified*, and *Wolbachia pipentis*, accounted for more than 99% of the total sequences between treated and untreated bed bugs. This is even though the number of sequences of dominant genera in untreated bed bugs is relatively higher than the number of sequences of treated bed bugs. Table 2 displays a distinct differentiation in the number of sequences between the two replicates used for control (C-C2), chlorfluazuron (CH-CH2) and β-cyfluthrin + imidacloprid (TM1-TM2) due to low quality of DNA during sample preparation.

DISCUSSION

This study focuses on the characterisation of the bacterial community between insecticidal-treated and untreated tropical bed bugs, *C. hemipterus,* employing sequencing via high-throughput. Adaptation of an organism to environmental conditions may induce an amendment in physiological function, which triggers modification in the regulation of its microbiome constituents. Symbionts play a key role in maintaining the wellness of their host as they themselves depend on the host to gain food and shelter. The study displays Proteobacteria as the predominant phylum in both treated and untreated bed bugs. The dominance of proteobacteria (> 99%) at the phylum level shows no significant difference across the

control and untreated samples. Hence, the genus level was observed (Fig. 4) to have a better illustration of the symbiont's community variations.

However, the variability between each sample showcases less than 0.1% abundance with no distinct differentiation. The alpha-diversity in all samples was relatively low due to the samples used being of the same strain and receiving similar blood source, although the microbial interactions or insect immune systems plausibly affect the bacterial community's composition and proliferation rate (Boissière *et al.* 2012). Interestingly, this study exhibits variations between replicate to replicate, which could be justified due to technicality issue during sample preparation and analysis, whereby the DNA concentration may be a little different, although the concentration and purity were verified using a spectrophotometer thence gel electrophoresis should be employed for a convincing result. Besides, too much primer or template DNA could also potentially influence such results (Table 2).

The findings point out that the application of insecticides has a negligible impact on the overall diversity of the bacterial community harbouring tropical bed bugs. A similar result had supported this discovery, where Soh and Singham (2022) deduce that the diversity of the bacterial community between chemical + antibiotic fed bed bugs was similar to that of the untreated bed bugs. Nevertheless, despite no significant differences in alpha diversity, OTUs display a distinct differentiation in pyriproxyfen-treated bed bugs compared to other tested samples. Theoretically, control and treated samples should display a distinct differentiation in their microbial composition. However, this study overall reveals there were no significant dissimilarities in the microbial composition except for the pyriproxyfen-treated sample.

This may be due to the method of treatment employed, which was surface contact that requires the bed bugs to sit on the insecticides in order for them to pick up the chemical and absorb via their cuticle to reach their system. Thus, the effect of the insecticide could be minimal in influencing the microbial composition within *C. hemipterus*. The disclosure of bacterial genera that were present in all samples indicated that they were transmitted vertically; furthermore, their existence is vital to their hosts as they supply nutrients and support homeostasis as well as insect immunity (Hypša & Aksoy 1997; Lim & Majid 2021). Among the shared bacteria, members of Proteobacteria, *Wolbachia* and *Burkholderia-Caballeronia-Paraburkholderia*. predominates the bacterial constituent of *C. hemipterus*. Although the proteobacterium found in these studies was labelled as unclassified, it is common to find alpha and gamma-proteobacteria in *C. hemipterus*, which aligns with the findings of Hypša and Aksoy (1997).

Wolbachia are an indisputable endosymbiont of *Cimex* spp. as this fauna resides within the bacteriome of *C. hemipterus* and acts as a contractual provider of nutrients – vitamin B, biotin (B7), riboflavin (B2), aside from being liable for host reproduction (Hosokawa *et al.* 2010; Akhoundi *et al.* 2016; Bellinvia *et al.* 2020; Lim & Majid 2021). Fascinatingly, *Wolbachia* were found to be capable of improving both male and female bed bugs' fitness by boosting egg laying and hatching rate, improving progeny body size, emergence succession and lowering wound or infection due to episodes of traumatic insemination (Heaton 2013; Hickin *et al.* 2022). This study theoretically managed to classify *Wolbachia* into two strains- *Wolbachia unclassified* and *Wolbachia pipientis,* where two strains mark up the plethora of gram negative alphaproteobacterial. The second most prevalent genus in this study was *Pectobacteria*, a plant-hazardous enterobacteria that causes economically significant soft rot disease (Amir *et al.* 2020; Hugouvieux-Cotte-Pattat 2016). This gram-negative gamma proteobacterium is likely to be represented as the secondary endosymbiont of the bed bugs (Pietri *et al.* 2020). Regardless, loss of *Wolbachia* and *Pectobacteria* in bed bugs has signified deceleration in development time and shrinkage in body size, though it was not refined; the growth retardation was induced by these bacteria (Goodman 2016).

Application of insecticides via contact bioassay typically affects the regulation of bacterial symbiont, as microbial production is either upregulated or downregulated when compared with the untreated bed bug sample. *Burkholderia-Caballeronia-Paraburkholderia* were reported as maternally transmitted strains that are common in insect pests; they were certainly present in all samples except control (C1) and tebufenozide (T1) in this study (Peta & Pietri 2021). But by far, there are no validated findings to dictate the presence of *Burkholderia-Caballeronia-Paraburkholderia* in arthropods. Based on this study, genus Treponema was found in control (C1), chlorfluazuron (CH1) and tebufenozide (T1) treated samples. The Treponema of the spirochetes class is usually found in humans, animals and insects. Exploration of biochemistry and genomic assay reveals this genus has a distinctive function in termite species where they inherently stimulate nutritional balance, energy catabolism, and hydrogen scavenging in the insect gut (Evans *et al.* 2011; Diouf *et al.* 2018). *Sanguibacter-flavimobilis* is a soil-based facultative aerobic bacterium that was exceptionally identified from a tebufenozide-treated bed bug sample (Dueholm *et al.* 2022). The presence of this genus was customarily detected from the blood of healthy cows, beef cattle and Tenebrio molitor, but the sequence of reads was doubtlessly low (Tye 2023). Bed bugs in this study may have incorporated with these genera via horizontal contact of the environmental biota. From the overall taxonomic classification, could be deduced that there was no human pathogenic bed bug symbiont discovered. Basically, the symbionts identified were

commonly found in the environment. Nevertheless, the Burkholderia genus was reported to be found within the human gut as an opportunistic pathogen, but the clade *Burkholderia-Paraburkholderia-Cabelleronia* could be classified as novel within bed bugs, not to mention in humans (Mahenthiralingam *et al.* 2008)

Insecticides treated with different modes of action do not signify a distinct up- or downregulation of endosymbionts when compared to controls. However, only Juvenile hormone analogue (JHA) substantially modulates the evenness of gut mycobiome community in pyriproxyfen-treated bed bugs, where the titer of *Burkholderia-Caballeronia-Paraburkholderia* was relatively higher, as Li *et al.* (2021) discloses abundance of normal flora tends to decrease, but the secondary flora began to increase from the JHA treatment (Lu *et al.* 2022). Besides, host-driven mechanisms also could potentially induce shifts in the composition of bed bug bacterial communities, where the host may create altered antimicrobial proteins, pH changes, decreased mucus secretion on the intestinal lining and decreased intestine size throughout the study period (Kohl *et al.* 2014).

CONCLUSION

This study examined the bacterial community structure of *C. hemipterus* in two groups: one that was treated with insecticides and another that was left untreated. There was no discernible difference in the bacterial community makeup between the treated and untreated samples. Nevertheless, insecticides may have a slight effect on the titer of gut symbiont production in the treated sample. Although the roles of some of the bacteria found in tropical bed bugs are unknown, the results of this study help shed light on the possible roles of most bacteria in bed bugs. It is necessary to conduct additional research into the physiological characteristics, potential functions, and interactions of these bacteria with bed insect biology.

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AUTHORS' DECLARATIONS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS DECLARATIONS

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Universiti Sains Malaysia Research Ethics Committee (Human) JEPeM, Code: USM/JEPeM/19120868

DATA AVAILABILITY STATEMENT

The manuscript has no associated data.

AUTHORS' CONTRIBUTIONS

Nurhidayah Taibukahn: Methodology, investigation, data curation, formal analysis, writing – original draft, writing – review and editing.

Martini Martini: Conceptualisation, supervision, writing – review & editing.

Abdul Hafiz Ab Majid: Conceptualisation, supervision, project administration, resources, funding acquisition, writing – review & editing.

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