Variable Gut Microbiota Profiles of Coffee Berry Borer (*Hypothenemus hampei* Ferrari, 1867) across Organic Coffee Plantation Habitats in Chiang Rai, Northern Thailand

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Summary

The coffee berry borer (CBB, *Hypothenemus hampei* Ferrari, 1867) is a coffee pest causing significant economic losses worldwide. The ability of the insect to withstand coffee toxicity has been attributed to its gut microbiota. In this study, CBBs were collected from three organic coffee plantations with variable environmental conditions in Chiang Rai, characterized by evergreen, shaded and open plantations. This research aimed to assess the abundance and diversity of the CBB microbiota in three coffee plantations and the dominant or specific bacterial taxa in each habitat. The CBB gut microbiota profiles were also compared across the three plantations. Next-generation sequencing revealed that the microbiota profiles of the CBBs from the evergreen and shaded coffee plantations had similar species richness, with 23 operational taxonomic units (OTU) in each. The gut microbiome profile of the CBBs from the opened organic coffee plantation was less similar to the other two and had lower diversity, with only 10 OTUs. The most abundant species found in this study was *Pseudomonas fulva* (51%), followed by *Achromobacter insolitus* (19%) and *Kluyvera cryocrescens* (12%), which are important for caffeine degradation. Our results of the gut microbial community of CBBs provide valuable baseline data on organic coffee in northern Thailand that can be used for coffee pest and insect control in the future.

Key words

Arabica coffee, environmental conditions, gut microbiota, *Hypothenemus hampei* Ferrari, 1867

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Introduction

Coffee berry borer, or CBB (*Hypothenemus hampei* Ferrari, 1867, Coleoptera, Scolytidae), is one of the most serious coffee pests worldwide (Ceja-Navarro et al., 2015; Johnson et al., 2020). This insect, native to Central Africa, can also be found throughout coffee-growing regions, causing an estimated annual economic loss of over $500 million (Vega et al., 2002). Male insects (1.0–1.5 mm) are slightly smaller than their female counterparts (1.4–2.0 mm). Females have well-developed wings and are capable of flying, while males have vestigial wings and are flightless (Jaruseranee et al., 2021). Females mate inside the coffee berry, then leave to find other coffee berries or habitats to deposit their eggs. Females penetrate the coffee berries and excrete their waste inside the fruits. These actions destroy the coffee berries, leaving them susceptible to infestation by fungi or bacteria. Larvae feed on the seed, reducing its quality and causing significant losses in yield (Ceja-Navarro et al., 2015). Coffee fruits may drop before harvesting, and coffee beans can often be of lesser quality upon harvest. As a result, CBB causes damage to both the quantity and quality of coffee fruit.

The gut microbiota comprise all microbes living in the gastrointestinal tract. These endosymbionts potentially provide numerous beneficial services to their hosts, including aiding in the molecular digestion of organic compounds (Engel and Moran, 2013), chemical detoxification (Ceja-Navarro et al., 2015; Zhao et al., 2022), plant compound detoxification (Zhang et al., 2022), protection against parasites and pathogens, as well as reproductive manipulations (Zhao et al., 2022). Recent studies have revealed that the diversity of gut bacteria is determined by several factors, with diet being the primary determinant and the environment (Disayathanoowat et al., 2020), host developmental stage (Mejía-Alvarado et al., 2021), and host phylogeny playing secondary roles (Yun et al., 2014; Li et al., 2022).

In insects, gut microbiota play crucial roles in various aspects of host nutrition, physiology and behaviour (Li et al., 2022; Zhao et al., 2022). Previous studies have highlighted the importance of bacterial-host coexistence and the long-term stability of the host-symbiont relationship under different environmental conditions (Li et al., 2022; Wang et al., 2022). For example, *Pseudomonas*, a major taxon in the microbiome, degrades caffeine in the gut of Arabica coffee pests. Given that coffee beans provide a substantial nutrition source, it can be suggested that the gut of coffee pests offers an ideal environment for a specific microbiome. These organisms can subsist on caffeine as their source of carbon and nitrogen (Filho and Mazzafera, 2003). Examples of caffeine-degrading bacteria include *Klebsiella* sp., *Rhodococcus* sp., *Stemphyllium* sp., *Serratia marcescens*, *Pseudomonas putida*, *Aspergillus tamarii*, and *Penicillium commune* (Gokulakrisnan et al., 2005).

The gut microbiome of CBB is both diverse and complex (Filho and Mazzafera, 2003; Vega et al., 2021). Previous studies based on culture-independent techniques have revealed that the digestive tracts of CBB encompass a wide variety of bacteria, mainly belonging to the phyla Proteobacteria, Firmicutes and Bacteroidetes (Mejía-Alvarado et al., 2021; Jaruseranee and Kamtaeja, 2023). The CBB gut microbial community plays an important role in the insect's ability to utilize coffee beans as a food source (Ceja-Navarro et al., 2015). An essential function of the CBB gut microbiome is the transformation of toxins into food sources. This process involves the breakdown of caffeine, a toxic alkaloid that would otherwise be harmful to CBB. This activity serves as a defense mechanism to inhibit infection by other insects. Caffeine degradation and the digestion of coffee bean components contribute significantly to shaping the gut microbiome of this insect (Ceja-Navarro et al., 2015).

Research on the microbial community associated with the CBB and understanding its implications for insect nutrition, defense and reproduction could lead to its potential for manipulation and, ultimately, leading to its biological control (Johnson et al., 2020). Understanding the effects of environmental conditions on the CBB microbiota could lead to effective strategies for managing CBB infestations. Specifically, the coexistence between the gut microbiome and insects is essential for insect survival and disrupting this relationship can be a potential target for pest management (Douglas, 2007). The bacterial microbiota in the CBB exhibit diversity and are influenced by the host's diet, which is related to coffee plant species and the geographical distribution of insect populations (Marino et al., 2018). Despite variations in microbiome composition demonstrated by biogeographic analysis, there is a shared core of microbiota among insect specimens, which includes members of Pseudomonadales, Enterobacteriales, Turicibacteriales, Rhizobiales, Alteromonadales and Actinomycetales (Ceja-Navarro et al., 2015). For example, bacteria such as *Pseudomonas*, *Sphingobacterium*, and *Stenotrophomonas* are known to degrade caffeine in the gut of the CBB. However, due to the lack of information on several taxa, more investigations are required, focusing on insect interaction and functional studies.

In this study, we focused on coffee plantations in Chiang Rai, the largest producer of Arabica coffee in Thailand. We assessed the bacterial communities associated with the CBB on coffee fruits in three different habitats: evergreen, shaded and open areas. We predicted that CBBs from the more natural evergreen and shaded coffee plantations would exhibit more diverse microbiota compared to CBBs from open area plantations, which undergo more manipulation. This study aimed to evaluate the diversity and abundance of CBB microbiota infecting these three coffee plantation habitats and identify dominant or specific bacterial taxa present in each habitat. Our characterization of the gut microbial community of CBB offers valuable baseline data for pest control strategies, particularly in the context of organic coffee cuiltivation in northern Thailand.

Materials and Methods

Coffee berry borer insects (*Hypothenemus hampei*, Coleoptera, Scolytidae) were obtained from infested Arabica coffee fruits from three organic plantations in Chiang Rai Province, northern Thailand. The characteristics of the plantations were as follows: 1) an evergreen organic coffee plot situated at Doi Pangkhon (19°53' N., 99°35' E.) at an altitude of 1400 m, the highest elevation of the study. The site is located on a hill covered with evergreen forest with dense vegetation at the sampling site (Fig. 1c), 2) a shaded organic coffee plot situated at Doi Pangkhon (19°54' N., 99°36' E.) at an elevation of 1200 m and located at a hillside area on a steep slope.

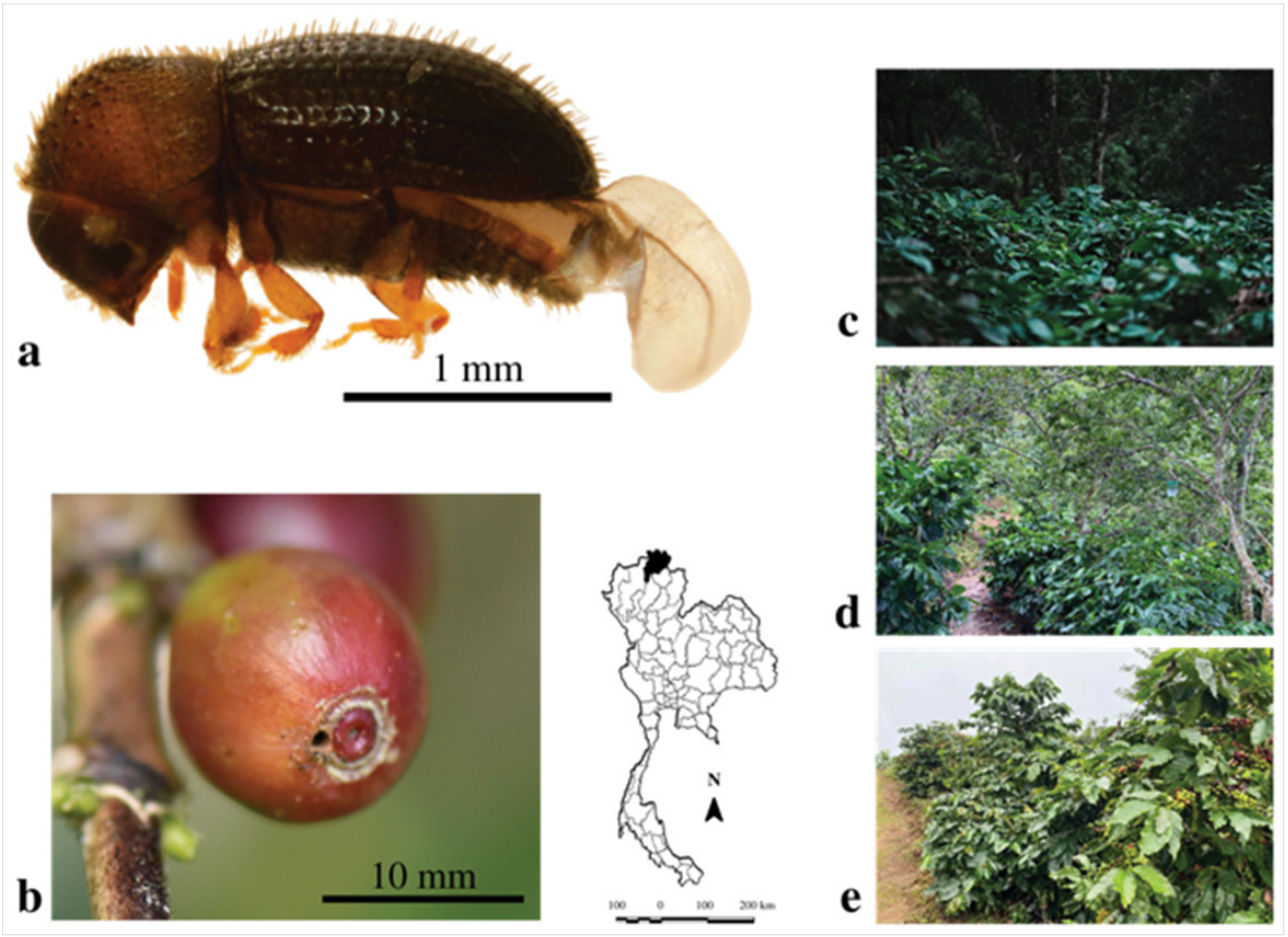

Figure 1. (a) Adult female of coffee berry borer (*Hypothenemus hampei* Ferrari). (b) Penetration (tiny hole) at the tip of the coffee berry. Map showing the location of the collecting sites of the evergreen (c), shaded (d) and opened area (e) organic coffee plantation habitats.

Arabica coffee trees are grown under shade along with other fruit trees such as *Prunus persica* (L.) Batsch and *Macadamia integrifolia* Maiden & Betche (Fig. 1d), and 3) an open area organic coffee plot situated at Doi Mae Mon (19°51' N., 99°36' E.) at an elevation of 1,000 m and located at an open hilltop plot, comprising only a monoculture of coffee in our study (Fig. 1e). From each plantation, two distant areas were randomly selected. Fifty insects per area were collected (100 insects per plantation, totalling to 300 insects). Samples were obtained between October and December 2021, when the berry of Arabica coffee was about to ripen.

Gut Microbial Culture: Insects were removed from the infested coffee fruits (Fig. 1a) and subjected to surface sterilization using 10% Clorox (NaOCl) solution for 10 min, followed by washing with sterile distilled water twice (Gruneck et al., 2022). Coffee berry borers were separated according to area and placed in tubes containing 200 mL of 1× phosphate buffer (Ceja-Navarro et al., 2012). Hence, there were six tubes, each containing 50 CBB. Insects were dissected and thoroughly macerated using a pellet pestle. Fifty microliters of the diluted gut solution from each tube were plated on agar plates containing mineral media (9.5mM KH_2PO_4, 4.8mM $MgSO_4$, 0.1mM $CaCl_2$, 0.8mM Na_2HPO_4 and 20 g L^{-1} bacto agar). The inoculated plates were incubated at 30 °C for one week. The growth of bacteria was monitored daily to follow the formation of morphologically distinct colonies. At the end of the week, all bacterial colonies from each plate were scraped, transferred to six separate microtubes containing 50% glycerol and kept at -20 °C until further use.

DNA Extraction: Genomic DNA was isolated using NucleoSpin®Microbial DNA Kit (Macherey-Nagel), following the gram-positive and gram-negative bacterial DNA extraction protocol. DNA concentration and quality were measured using a NanoDrop™ spectrophotometer (ThermoFisher Scientific) and visualized in 2% agarose gel electrophoresis. The final volume per tube was 25 µL with DNA concentrations ranging between 71.14–81.67 ng μL^{-1}.

PCR Amplification of 16S rRNA Gene Fragments: The V3 and V4 regions of the 16S rRNA gene were amplified by PCR using specific barcode primers (V3V4-F: 5'- CCT ACG GGN GGC WGC AG-3', V3V4-R: 5'- GAC TAC HVG GGT ATC TAA TC-3') (Klindworth et al., 2013). The final volume of the PCR mixture is 25 µL and comprises 12.5 µL of 2× KAPA HiFi HotStart ReadyMix (Macrogen, Inc.), 2.5 µL (5 ng μL^{-1}) of DNA, and 5 µl of each forward and reverse primers (1 µM stock solution). The temperature profiles were as follows: initial denaturation at 95 °C for 3 minutes, followed by 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds, with a final extension at 72 °C for 5 minutes. The expected size of the PCR products

was ~ 450 bp, which was verified using the Bioanalyzer (Agilent Technologies 2100). Libraries were generated using MiSeq reagent kits, quantified by the dsDNA binding dyes fluorometric method, and sequenced with an Agilent Technologies 2100 Bioanalyzer (Macrogen, Inc.).

16S rRNA Gene Amplicon-Sequencing Analysis: The obtained amplicons were purified from free primers and primer dimer by-products using AMPure XP beads. DNA was quantified using a fluorometer, which employs dsDNA binding dyes. MiSeq reporter software (MSR) was used for metagenomics analysis of the 16S rRNA data of V3 and V4 amplicons. Raw reads were filtered using QIIME (version 1.7.0). The classification was based on the NCBI database. The resulting taxa of this analysis were classified at the kingdom, phylum, class, order, family, genus, and species levels.

Diversity Analysis of Gut Microbiome: Gamma and beta diversity analysis was performed using QIIME (version 1.7.0). Beta diversity analysis was used to compare gut bacterial community structure between CBBs within and between coffee localities based on distance matrices implemented in QIIME (version 1.7.0). Gut bacterial diversity was measured by Chao1, Shannon index, and Gini-Simpson index in QIIME (version 1.7.0). Relative abundances at the phylum, genus, and species levels between subgroups were compared using Metastat by adjusting the false discovery rate (FDR). Variations in gut microbiota community structure and composition across subgroups were evaluated.

Results

Gamma Diversity: The gut bacterial communities in adult coffee berry borers (CBB) representing three coffee plantations were isolated and analysed. A total of 120,291 high-quality bacterial sequences were obtained from the three plantations. The number of sequences across samples ranged from 13,234 to 32,637. Read length across samples ranged from 439–466 bp. Reads were clustered into a total of 34 operational taxonomic units (OTUs). The identity of bacterial sequences to the top hit taxon in the database ranged from 84–100% (table 1). At the phylum level, gut bacterial isolates from adult CBB were dominated by Proteobacteria (29 species, 85%), followed by a small population of Bacteroidetes (3 species, 9%), Actinobacteria (1 species, 3%) and Cyanobacteria (1 species, 3%). Of these, 31 were gram-negative, while two (*Stenotrophomonas chelatiphaga* and *Curtobacterium oceanosedimentum*) were gram-positive bacteria. The 34 OTUs belonged to 12 families as follows: 10 OTUs of Pseudomonadaceae (*Pseudomonas fulva, P. putida, P. glareae, P. paralactis, P. plecoglossicida, P. proteolytica, P. qingdaonensis, P. reidholzensis, P. reinekei,* and *P. turukhanskensis*), seven OTUs of Enterobacteriaceae (*Cedecea lapagei, Citrobacter werkmanii, C. braakii, Kluyvera cryocrescens, Kosakonia quasisacchari, Phytobacter diazotrophicus,* and *Yokenella regensburgei*), four OTUs of Xanthomonadaceae (*Stenotrophomonas pavanii, S. chelatiphaga, S. maltophilia,* and S. *rhizophila*), four OTUs of Alcaligenaceae (*Achromobacter insolitus, A. kerstersii, A. pestifer,* and *Bordetella avium*; 2 OTUs of Yersiniaceae: (*Gibbsiella dentisursi* and *Serratia oryzae*), one OTU each in Brucellaceae (*Brucella pseudogrignonensis*), Pectobacteriaceae (*Pectobacterium atrosepticum*), Flavobacteriaceae (*Flavobacterium acidificum*), Muribaculaceae (*Muribaculum intestinale*), Sphingobacteriaceae (*Sphingobacterium siyangense*), Microbacteriaceae (*Curtobacterium oceanosedimentum*) and Oscillatoriaceae (*Aerosakkonema funiforme*).

Across the three organic coffee plantations, the most abundant species across all samples was *Pseudomonas fulva* (51%), followed by *Achromobacter insolitus* (19%) and *Kluyvera cryocrescens* (12%). *Stenotrophomonas rhizophila* (7%), *Brucella pseudogrignonensis* (4%), *Sphingobacterium siyangense* (2%), *Flavobacterium acidificum* (2%) and *Serratia oryzae* (1%) were less abundant. The other 26 species accounted for less than 1% of the community.

Alpha Diversity

Gut bacterial diversity was measured by OTUs, Chao1, Shannon index, and Gini-Simpson index in QIIME (version 1.7.0). The alpha diversity indices indicated that the gut microbiota diversity varied across the three coffee plantations (Fig. 2a, b, and c).

Evergreen Organic Coffee Plantation: The sequence read count ranged from 18,135 to 13,234. The total number of OTUs from this habitat was 23 species with a Chao1 index of 18.7 ± 0.9, indicating a high species richness. The diversity indices were moderately high in this habitat, as shown by the 1.3 ± 0.1 Shannon index and 0.5 ± 0.1 Gini-Simpson index. *Pseudomonas fulva* and *Kluyvera cryocrescens* dominated the bacterial community, representing 47% each. *Flavobacterium acidificum* (3%), *Pseudomonas proteolytica* (2%) and *Achromobacter insolitus* (1%) constituted smaller parts of the community. Seven species were mainly found in CBB from this plantation, *Citrobacter braakii, Kosakonia quasisacchari, Phytobacter diazotrophicus, Pseudomonas paralactis, P. putida, P. qingdaonensis,* and *Stenotrophomonas chelatiphaga* (Fig. 2a).

Shaded Organic Coffee Plantation: The sequence read count ranged from 21,650 to 22,912. The total number of OTUs was 23 species with a Chao1 index of 18.5 ± 4.2. The diversity indices of gut bacterial communities of shaded coffee CBB were highest in this habitat with a Shannon index of 1.7 ± 0.3 and Gini-Simpson index of 0.7 ± 0.1. The most dominant species found in shaded coffee was *Pseudomonas fulva* (52%), followed by *Kluyvera cryocrescens* (24%), *Achromobacter insolitus* (10%), *Flavobacterium acidificum* (8%) and *Serratia oryzae* (6%). *Achromobacter kerstersii, Achromobacter pestifer, Citrobacter werkmanii, Muribaculum intestinale, Pseudomonas reidholzensis, Pseudomonas turukhanskensis, Serratia oryzae,* were unique to this plot (Fig. 2b).

Open Area Organic Coffee Plantation: The sequence read count ranged from 21,289 to 23,071. The total number of OTUs was ten species with a Chao1 index of 8.0 ± 0.0. The diversity indices of gut bacterial communities of CBB in open area organic coffee were lowest in this habitat with Shannon index of 1.5 ± 0.1 and Gini-Simpson of 0.6 ± 0.0. The three most dominant bacteria in this plot were *Stenotrophomonas rhizophila* (38%), *Achromobacter insolitus* (26%), and *Brucella pseudogrignonensis* (23%). Small proportions of *Sphingobacterium siyangense* (9%), *Flavobacterium acidificum* (2%), and *Stenotrophomonas pavanii* (2%) were also found. *Curtobacterium oceanosedimentum* and *Stenotrophomonas maltophilia* were exclusively isolated in sun-exposed coffee plots (Fig. 2c).

Table 1. List of gut microbiota isolated from the gut of coffee berry borers

Family	Species	Identity (%)	Habitat	Abundant (%)
Pseudomonadaceae	*Pseudomonas fulva*	93-100	E, S	51%
Alcaligenaceae	*Achromobacter insolitus*	91-100	E, S, O	19%
Enterobacteriaceae	*Kluyvera cryocrescens*	96-100	E, S	12%
Xanthomonadaceae	*Stenotrophomonas rhizophila*	89-99	E, S, O	7%
Brucellaceae	*Brucella pseudogrignonensis*	99	E, S, O	4%
Yersiniaceae	*Serratia oryzae*	98	S	1%
Alcaligenaceae	*Achromobacter kerstersii*	93-94	S	<1%
Alcaligenaceae	*Achromobacter pestifer*	93	S	<1%
Alcaligenaceae	*Bordetella avium*	93-94	E, S, O	<1%
Enterobacteriaceae	*Cedecea lapagei*	93	E, S	<1%
Enterobacteriaceae	*Citrobacter braakii*	97	E	<1%
Enterobacteriaceae	*Citrobacter werkmanii*	96	S	<1%
Enterobacteriaceae	*Kosakonia quasisacchari*	95	E	<1%
Enterobacteriaceae	*Phytobacter diazotrophicus*	96	E	<1%
Enterobacteriaceae	*Yokenella regensburgei*	92-97	E, S	<1%
Pectobacteriaceae	*Pectobacterium atrosepticum*	94	E, S	<1%
Pseudomonadaceae	*Pseudomonas glareae*	95-97	E, S	<1%
Pseudomonadaceae	*Pseudomonas paralactis*	93	E	<1%
Pseudomonadaceae	*Pseudomonas plecoglossicida*	99	E, S	<1%
Pseudomonadaceae	*Pseudomonas proteolytica*	99	E, S	<1%
Pseudomonadaceae	*Pseudomonas putida*	99	E	<1%
Pseudomonadaceae	*Pseudomonas qingdaonensis*	93	E	<1%
Pseudomonadaceae	*Pseudomonas reidholzensis*	92	S	<1%
Pseudomonadaceae	*Pseudomonas reinekei*	95	E, S	<1%
Pseudomonadaceae	*Pseudomonas turukhanskensis*	97	S	<1%
Xanthomonadaceae	*Stenotrophomonas chelatiphaga*	94	E	<1%
Xanthomonadaceae	*Stenotrophomonas maltophilia*	94	O	<1%
Xanthomonadaceae	*Stenotrophomonas pavanii*	98	E, S, O	<1%
Yersiniaceae	*Gibbsiella dentisursi*	94	E, S	<1%
Sphingobacteriaceae	*Sphingobacterium siyangense*	99	E, O	2%
Flavobacteriaceae	*Flavobacterium acidificum*	99	E, S, O	2%
Muribaculaceae	*Muribaculum intestinale*	92	S	<1%
Oscillatoriaceae	*Aerosakkonema funiforme*	86-87	E, S, O	<1%
Microbacteriaceae	*Curtobacterium oceanosedimentum*	99	O	<1%

Note: **Identity:** percentage of OTUs indentity blasted in NCBI database.
Habitat: E: evergreen, S: shaded, and O: epened-area.
Abundant: percentage of OTUs abundant across all three habitats.

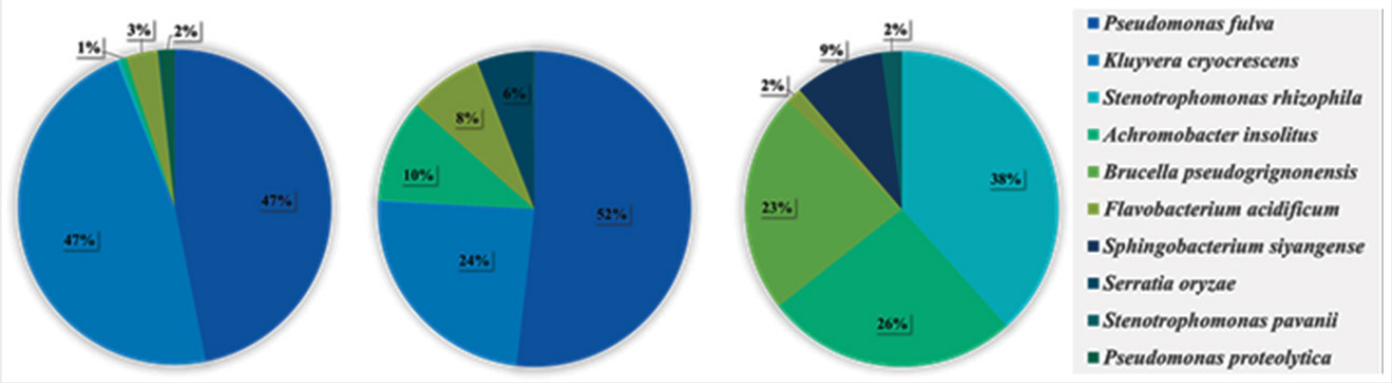

Figure 2. Comparison of species abundance isolated in each organic coffee habitat; evergreen (a), shaded (b), and opened area (c).

Beta Diversity

Evergreen* × *Shaded Organic Coffee Plantations: A total of 31 OTUs were isolated from these two habitats. The number of OTUs was equal between evergreen (23 species) and shaded coffee (23 species) plantations. Species diversity indices of the CBB microbiota from shaded coffee (Shannon index = 1.7 and Gini-Simpson = 0.7) were higher than those from evergreen organic coffee (Shannon index = 1.3 and Gini-Simpson = 0.5). The samples from these two environments shared 17 species (50%): *Achromobacter insolitus, Aerosakkonema funiforme, Bordetella avium, Brucella pseudogrignonensis, Cedecea lapagei, Flavobacterium acidificum, Gibbsiella dentisursi, Kluyvera cryocrescens, Pectobacterium atrospecticum, Pseudomonas fulva, P glareae, P. plecoglossicida, P. proteolytica, P. reinekei, Stenotrophomonas pavanii, S. rhizophila,* and *Yokenella regensburgei* (Fig. 3).

Shaded* × *Open Area Coffee Plantations: A total of 27 OTUs were isolated from these two habitats. The number of OTUs was higher in the CBB microbiota from the shaded plantation (23 species) than the open area (10 species). The samples from these two environments shared seven species (21%): *Achromobacter insolitus, Aerosakkonema funiforme, Bordetella avium, Brucella pseudogrignonensis, Flavobacterium acidificum, Stenotrophomonas pavanii* and *S. rhizophila*.

Evergreen* × *Open Area Coffee Plantations: A total number of 27 OTUs were isolated from these two habitats. The number of OTUs was higher in the microbiota of CBBs from evergreen coffee (23 species) than those from open area coffee plantations (10 species). The samples from these two environments shared eight species (24%): *Achromobacter insolitus, Aerosakkonema funiforme, Bordetella avium, Brucella pseudogrignonensis, Flavobacterium acidificum, Sphingobacterium siyangense, Stenotrophomonas pavanii* and *S. rhizophila*.

Discussion

The presence of a bacterial gut microbiome in Coffee Berry Borers across diverse organic coffee plantations highlights the insect-bacteria symbiotic relationship. The gut bacterial species of CBB play important roles in metabolic processes, such as food digestion and the detoxification of caffeine (Mejía-Alvarado et al., 2021). In this study, we isolated and cultured gut microbiomes from coffee plantations with different characteristics, and observed habitat-dependent species abundances. Across the three coffee habitats examined, a differential abundance of specific taxa within the gut microbiota was noted. In general, evergreen and shaded organic coffee habitats shared more bacterial taxa. This similarity in diversity and composition of bacterial microbiota in the CBBs from these two coffee habitats likely reflects their environmental homogeneity. In both habitats, *Pseudomonas fulva* and *Kluyvera cryocrescens* were dominant, and these taxa are known for their important roles in caffeine detoxification in several habitats (Ceja-Navarro et al., 2015; Marino et al., 2018). In contrast, open area plots harbored different types of bacteria, with *Stenotrophomonas rhizophila*, *Achromobacter insolitus*, and *Brucella pseudogrignonensis* being the three most common species. These results suggest that the CBB microbiota adapt to local conditions, varying according to the specific characteristics of the coffee plantation plots.

Pseudomonas, in general, emerged as the most common bacterial group in the cultured microbiome of coffee insects (Ceja-Navarro et al., 2015; Marino et al., 2018; Summers et al., 2015). *Pseudomonas fulva* was the most abundant species in evergreen and shaded coffee plots. This particular species has been reported to play a crucial role in the caffeine degradation mechanism for its host, the Coffee Berry Borer (Ceja-Navarro et al. 2015). This ability allows the CBB insect to survive inside the coffee berry without experiencing any toxic effects. This bacterium expresses the *ndmA* gene, responsible for encoding the alpha subunit of caffeine demethylase, contributing significantly to the caffeine detoxification ability of *P. fulva* (Ceja-Navarro et al. 2015). The species was found to be caffeine-tolerant, exhibiting significant growth in high-caffeine environments, which suggests its potential role in the caffeine-related mechanisms of CBBs (Vega et al., 2021; Jaruseranee and Kamtaeja, 2023).

In our study, *P. putida* was also isolated, but at a lower abundance level (< 1%) in the evergreen organic habitat and was not found in the other two habitats. This species has been reported as an important caffeine-degrading organism in plantations across North America, South America, Europe and Asia (Summers et al., 2015). Studies focusing on the metabolic potential of *P. putida* have revealed that this bacterium employed N-demethylation catabolic pathways to break down caffeine, resulting in the production of carbon dioxide and ammonia (Summers et al., 2015).

Figure 3. Species network showing distribution of microbial taxa across all three organic coffee habitats

Kluyvera cryocrescens is a gram-negative bacterium dominant in the microbiota of CBBs from evergreen and shaded coffee plantations. *Kluyvera cryocrescens*, initially discovered in the gut of CBBs, possesses cellulose-degrading and nitrogen-fixation abilities, as previously reported. The metabolic by-products of these reactions include nitrogenous compounds essential for amino acid synthesis, indicating the role of this gut resident in the host's nutrition (Wang et al., 2022).

Kluyvera has also been reported to be significantly abundant in the gut of various organisms such as grasshoppers, red firebugs (*Pyrrhocoris apterus*) and populations of *Locusta migratoria manilensis* reared with high amounts of cellulose sources like goosegrass, maize leaves, and pakchoi (Wang et al., 2022; Sudakaran et al., 2012). Based on this information, it can be hypothesized that *K. cryocrescens* may play an important role in the survival of CBBs, particularly in denser vegetation environments.

Differences in growing conditions and altitude not only resulted in widely varying nutritional profiles in coffee fruits (Husni et al., 2022), but also provided preferable conditions for some groups of gut bacterial species. For example, *Pseudomonas, Pantoea, Enterobacter,* and *Stenotrophomonas* were identified, representing species that prefer warmer temperatures (Marino et al., 2018). The results of this study imply that environmental factors, such as elevation and habitat characteristics, greatly influence the gut bacterial community. The study areas of evergreen and shaded organic coffee are located at high elevations

of 1400 m and 1200 m, respectively, where temperatures and light intensity are lower compared to the open area coffee plantation. In the northern region of Thailand where the research took place, elevation has a significant impact on the characteristics of the habitat. The vegetation or crown cover is denser in the evergreen and shaded habitats compared to the open one. The open area coffee plots, located at 1000 m elevation in this study, have higher maximum temperatures than the shaded coffee plots. Thus, the lower levels of gut microbial species identified in the open habitat may be attributed to differences in environmental conditions, such as temperature, light intensity and moisture.

The species that dominate in the open study area are *Stenotrophomonas rhizophila, Achromobacter insolitus,* and *Brucella pseudogrignonensis*, all of which are newly discovered in the gut bacterial profiles of CBBs. Despite limited knowledge about their ability to perform caffeine oxidation and other metabolic activities, few studies have explored the diverse endosymbionts isolated from CBB, particularly in naturally occurring plantations influenced by sunny and shaded conditions. *Stenotrophomonas* spp., a gram-negative bacterium, has been found to degrade caffeine and cellulose in various types of insects (Arias-Cordero et al., 2012; Ceja-Navarro et al., 2015). The genus *Achromobacter* are obligate aerobes and common inhabitants of insects that have been reported in culture-dependent studies from the guts of European forest cockchafer (*Melolontha hippocastani*) (Arias-Cordero et al., 2012), and scarab beetles (*Pachnoda ephippiata* and *P. marginata*) (Andert et al., 2010). *Brucella pseudogrignonensis* is a gram-negative, non-motile bacteria. One species in this genus, *B. melitensis*, has been reported as pseudo-endosymbionts or pathogenic bacterial communities from several species of plant sap-sucking insects, such as aphids (Baumann, 2005).

The CBBs, similar to other coffee pest insects, were dependent on their gut microbiota for essential functions such as digestion, defense against pathogens, immunological control, and the breakdown of toxic compounds. The study has revealed that species abundances vary across different coffee plantation characteristics, with specific taxa of gut microbiota identified. Future experiments on the metabolic functions of the microbiota could further enhance our understanding of their role in the distribution of CBBs and their contribution towards more effective pest-insect control strategies. This research provides important information for organic coffee plantations in northern Thailand by shedding light on the symbiotic relationship between bacteria and insects.

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Ethical Approval

This research was authorized under permission from the Research Ethics Review Committee of Mae Fah Luang University (ethics license number: AR03/64).

CRediT authorship contribution statement

Nanthnit Jaruseranee: The grant holder and project owner, carried out the majority of the experiments and edited the manuscript. **Somboon Kamtaeja:** Performed research and conceptual investigation, field collection, data analysis, Original draft preparation.

Declaration of Competing Interest

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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