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Caffeine Concentration Effects on Gut Microbiota of the Coffee Berry Borer (*Hypothenemus hampei* Ferrari) and Possible Links to Insect Pest Control

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Abstract

Coffee Berry Borer (CBB) is a significant coffee pest worldwide. This includes Thailand. The arabica coffee plantations in Chiang Rai province, northern Thailand, have experienced significant economic losses as a consequence of this insect. The purpose of this study was to investigate changes in the gut bacterial community in response to caffeine concentrations in the growing environment, which may have implications for coffee pest insect control. A total of 38 species of gut microbiota were grown in non-caffeine conditions. On average, 14.5 ± 1.8 species were found in all 6 sampling sites. In caffeine conditions, the number of culturable gut bacteria was significantly decreased to 18 species with an average species abundance of 7.2 ± 3.1. The results revealed that *Pseudomonas fulva* and *P. punonensis* were caffeine-tolerant species that displayed significant growth at 20 mM caffeine concentration. The findings of this study will contribute to a better understanding of gut microbiota in CBBs and its potential application for coffee insect pest control, as well as valuable information on organic coffee from northern Thailand.

Keywords: Caffeine, *Coffea arabica*, Coffee pest control, Gut bacteria, *Hypothenemus hampei*, 16S rRNA gene

Introduction

Caffeine ($C_8H_{10}N_4O_2$ or 1,3,7-trimethylxanthine) is an alkaloid found in coffee plants (genus *Coffea*), and constitutes a chemical defense mechanism that protects against insect pests [1,2]. More specifically, caffeine is a secondary metabolite formed by purine nucleotides. Caffeine levels in coffee beans vary across species of the genus *Coffea*. For example, in the arabica coffee beans (*C. arabica*), caffeine ranged from 12.2 - 12.8 mg g^{-1} of dry weight, whereas for those of robusta coffee beans (*C. canephora*) the caffeine concentration was higher, ranging from 13.1 - 17.4 mg g^{-1} of dry weight [3]. Caffeine has also been detected in coffee pulp. For example, in *C. arabica* pulp, caffeine was 0.26 %, while in *C. canephora* pulp, it was 0.12 % [4]. Caffeine is a natural product that is also found in cacao beans, kola nuts, guarana berries, and tea leaves [5]. Caffeine is bitter tasting and acts as a defense mechanism against herbivorous insects. The alkaloid also causes insect paralysis and nervous system toxicity, by inhibiting phosphodiesterase activity and producing intracellular AMP [6]. Hence, the chemical toxicity mechanism of caffeine is critical for preventing pest outbreaks in *Coffea* and other plants of economic importance.

Insect symbionts are microorganisms residing in the insect gut and have been shown to be capable of converting toxic caffeine into non-toxic chemical compounds. Caffeine degradation bacteria, such as *Pseudomonas*, *Klebsiella* and *Pantoea*, have functional roles in oxidizing caffeine (or 1, 3, 7-trimethylxanthine) to xanthine [7,8]. Xanthine is a non-toxic organic compound that also serves as a carbon-nitrogen source for coffee insects [9]. Caffeine degrading gut microorganisms can survive on caffeine as a source of carbon and nitrogen [3]. Previously, more than 35 species of caffeine-degrading bacteria were isolated from insect guts, including *Pseudomonas fulva*, *Pantoea eucalypti*, *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Brachybacterium rhamnosum* [1,10]. Caffeine-degrading bacteria also include *Klebsiella* sp., *Rhodococcus* sp., and *Stemphyllium* sp. [1,7]. Of the above, *Pseudomonas* is the major caffeine-degrading microbiome taxon in the gut of arabica coffee pests [1].

Coffee Berry Borer, or CBB, (*Hypothenemus hampei* Ferrari; Coleoptera: Scolytidae), is the most serious coffee pest worldwide [10,11]. This pest has evolved to be caffeine resistant, allowing it to survive and reproduce inside coffee fruits [12]. The insect is sexually dimorphic. Males are very small with a 1.0 - 1.5 mm body length, and flightless due to very small remnant wings. Females are 1.4 - 2.0 mm in body length and have well-developed wings [13]. A sexual bias ratio of 38:1 of females to males has been reported in coffee fruits [12]. Females bore a tiny hole at the tip of the coffee berry, which they use to enter and deposit eggs in galleries within the berry. After penetration, the female can directly destroy the coffee beans and this is the primary cause of infestation by fungi or bacteria. The total number of laying eggs per mating session per female is approximately 30 - 70. Larvae feed on the seed of the berry. Coffee fruits may drop prior to harvesting, and coffee bean quality may be reduced [9,11]. The resulting losses are of both a quantitative and qualitative nature for the final product.

In this study, the influence of caffeine on the gut microbiome of the CBBs was studied in a laboratory. The gut microbiota of adult CBBs was obtained by collecting penetrated and ripped coffee fruits growing in coffee habitats in Chiang Rai province, north of Thailand. The bacterial community changes in response to caffeine concentrations were explored in the growing environment. The purpose of this experimental study was to investigate which gut microbial species dominated in a high caffeine environment versus normal media, and which species ceased growing in high caffeine concentration experiments. This research identified symbiont species in CBBs. The findings of this study will contribute to a better understanding of gut microbiota in CBBs and its potential application for coffee pest insect control by using plant extracts (like caffeine) that cease the intestinal microorganisms, as well as valuable information on organic coffee from northern Thailand.

Materials and methods

Gut microbial culture

Coffee berry borer insects were obtained from infested arabica coffee fruits from Doi Pangkhon (19°53' N., 99°35' E.; elevation 1,200 to 1,400 m.), Chiang Rai, northern Thailand. Doi Pangkhon is a shaded organic arabica coffee plantation. Arabica coffee trees are grown under shade along with other types of fruit trees such as *Prunus cerasoides* D.Don, *Prunus persica* (L.) Batsch and *Macadamia integrifolia* Maiden & Betche. Samples were obtained between October-December 2021. Samples were randomly collected from 6 areas of the study sites. At each collecting site, a total of fifty insects per area were collected. These were obtained from 10 arabica coffee plants, with 5 fruits of each plant being harvested. Insects were removed from the infested coffee fruits and surface sterilized with 10 % NaOCl (Clorox solution) for 10 min before being washed twice with sterile distilled water [14]. Coffee berry borers were divided into 6 tubes from 6 different areas. Then, CBB specimens were prepared to obtain gut solutions by dissecting and macerating in 200 mL of 1× phosphate buffer [8]. The gut solution from each location was cultured in 2 different caffeine concentrations.

In the 1st experiment, media with no caffeine were used. Fifty microliters of the gut solution were plated on agar plates containing normal mineral media: 9.5 mM KH_2PO_4, 4.8 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.8 mM Na_2HPO_4 and 20 g L^{-1} Bacto agar. In the 2nd experiment, media with 20 mM caffeine were used. Fifty microliters of the gut solution were plated on agar plates containing normal mineral media with caffeine: Mineral media (9.5 mM KH_2PO_4, 4.8 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.8 mM Na_2HPO_4 and 20 g L^{-1} Bacto agar) and 20 mM of caffeine.

All inoculated plates were incubated at 30 °C for 1 week. The growth of bacteria was monitored daily for the formation of colonies. All cultured bacterial colonies were scraped and transferred to a 1 mL microtube.

DNA extraction

Within a week, gut bacteria had fully grown on all inoculating plates. The scraping technique was used to remove the lawn of gut bacteria and transfer it into a 1.5 mL microtube. The NucleoSpin®Microbial DNA Kit (Macherey-Nagel) was used to isolate total genomic DNA following the protocol for Gram-positive and Gram-negative bacterial DNA extraction. A NanoDrop™ spectrophotometer (ThermoFisher Scientific) and 2 % agarose gel electrophoresis were used to measure DNA quality and concentration. Our final volume was 25 µL. The DNA concentration ranged between 71.1 - 81.7 ng μL^{-1}.

PCR amplification of 16S rRNA gene fragments

PCR with specific barcode primers (V3V4-F: 5′- CCT ACG GGN GGC WGC AG-3′, V3V4-R: 5′-GAC TAC HVG GGT ATC TAA TC-3’) was used to amplify the V3 and V4 regions of the 16S rRNA gene [15]. A 2x KAPA HiFi HotStart ReadyMix was used for all PCR reactions (Macrogen, Inc.). The final volume of each reaction was 25 µL, of which 12.5 µL was HotStart ReadyMix solution, 2.5 µL microbial DNA (5 ng μL^{-1}), and 5 µL of each forward (1 µM) and reverse primers (1 µM). Cycling conditions were as follows: 95 °C for 3 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for

30 s, and elongation at 72 °C for 30 s, with a final extension of 72 °C for 5 min. The expected size of PCR products was ~450 bp which was verified by using Bioanalyzer (Agilent Technologies 2100). The MiSeq reagent kits were used for library construction and dsDNA binding dye fluorometric method was used for quantification. Products were sequenced using an Agilent Technologies 2100 Bioanalyzer (Macrogen, Inc.). The MiSeq reporter software (MSR) was used for metagenomics analysis of the resulting 16S rRNA data. Raw reads were filtered using QIIME (version 1.7.0) and classified according to the NCBI database at the kingdom, phylum, class, order, family, genus, and species levels.

Diversity analysis of the gut microbiome

QIIME (version 1.7.0) was used to analyze species abundance of gut bacteria. The gut bacterial community structure from no caffeine versus caffeine conditions was compared. Relative abundances at phylum, family and species levels were also compared between the 2 conditions. Variations in gut microbiota community structure and composition across subgroups were evaluated.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics version 20 (SPSS, Inc.). The Levene's test for equal variances was assumed (F-test = 0.815, $p > 0.05$). Independent T-test was used to compared the differences between group, non-caffeine and 20 mM caffeine condition.

Ethical approval

The research for this study was authorized under permits from the research ethics review committee of Mae Fah Luang University (ethics license number: AR03/64).

Results and discussion

In this study, the caffeine effect on the gut microbiota community of CBBs was explored. Gut bacterial communities were obtained from adult CBBs and cultured at 0 and 20 mM of caffeine. Next generation sequencing of the 16S rRNA gene was performed to estimate the gut microbiome taxonomic abundance. We obtained 318,483 high-quality bacterial sequence reads. Considering all samples, the number of sequences ranged from 13,234 to 32,637 sequences. The total bases per condition analyzed in this study were 15,995,279 - 28,572,055 bp. The read length across our experiments ranged from 439 - 466 bp. The obtained reads clustered into 38 operational taxonomic units (OTUs). Blast searches against the NCBI database were performed for all sequences in this study with the OTU identity ranging from 92 - 100 % (**Table 1**).

In the non-caffeine condition, the average number of species found at all 6 sampling sites was 14.5 ± 1.8. Species abundance was significantly decreased (T-test = 5.807, $p < 0.000$) in the 20 mM caffeine condition at 7.2 ± 3.1 (**Figure 1**). The noted decrease was in the range of 53 %, with 18 species being the same as in the caffeine condition and 20 species being absent. The results revealed that the genus *Pseudomonas* (family Pseudomonadaceae) was dominant in the gut of CBBs, with *P. fulva* and *P. punonensis* being distinctly dominant in the caffeine condition. Hence, these 2 bacteria can be considered as caffeine-tolerant and important caffeine-degrading species in the gut of CBBs.

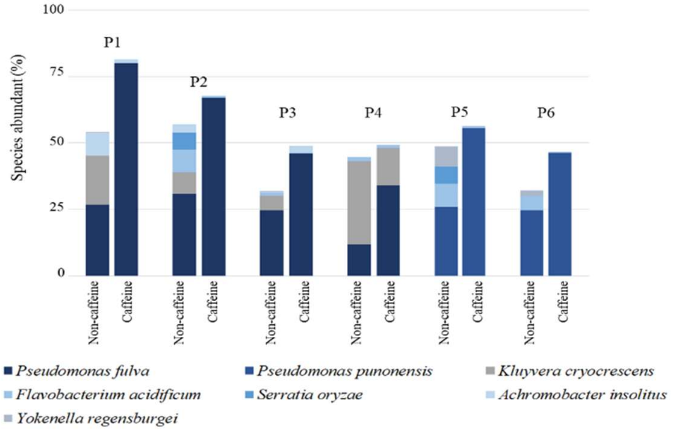

Figure 1 Species abundance of non-caffeine and caffeine conditions at all 6 sampling sites (P1 to P6), with distinct increase in the abundance of *P. fulva* and *P. punonensis*.

Gut bacteria communities cultured in mineral media + 0 mM caffeine

Thirty-seven species of Proteobacteria spanning 11 families and 1 species of Bacteroidetes from 1 family were isolated (**Table 1** and **Figure 2**). The most dominant gut bacteria belonged to Pseudomonadaceae (14 species: *Pseudomonas fulva*, *P. punonensis*, *P. flavescens*, *P. fluorescens*, *P. glareae*, *P. paralactis*, *P. plecoglossicida*, *P. proteolytica*, *P. putida*, *P. qingdaonensis*, *P. reidholzensis*, *P. reinekei*, *P. sihuiensis*, and *P. turukhanskensis*). The 2nd most dominant taxa were from Enterobacteriaceae (7 species: *Kluyvera cryocrescens*, *Yokenella regensburgei*, *Cedecea lapagei*, *Citrobacter braakii*, *C. werkmanii*, *Kosakonia quasisacchari*, and *Phytobacter diazotrophicus*, followed by Alcaligenaceae (4 species: *Achromobacter insolitus*, *A. deleyi*, *A. pestifer*, and *Bordetella avium*), Xanthomonadaceae (3 species: *Stenotrophomonas chelatiphaga*, *S. pavanii*, and *S. rhizophila*), Erwiniaceae (*Pantoea cypripedii* and *Pantoea wallisii*), family Yersiniaceae (*Gibbsiella dentisursi* and *Serratia oryzae*), Flavobacteriaceae (*Flavobacterium acidificum*) Brucellaceae (*Brucella pseudogrignonensis*), Muribaculaceae (*Muribaculum intestinale*), Pectobacteriaceae (*Pectobacterium atrosepticum*), Rhizobiaceae (*Rhizobium nepotum*) and Sphingobacteriaceae (*Sphingobacterium siyangense*).

Table 1 Species identity from 16S rRNA gene, and a result of caffeine concentrations to relative abundance of CBB gut bacteria.

Family	**Species**	**Identity**	**Relative abundance (%)**	
		(%)	**0 mM caff.**	**20 mM caff.**
Pseudomonadaceae	*Pseudomonas fulva**	100	34.18	64.15
Pseudomonadaceae	*P. punonensis**	100	18.39	28.80
Pseudomonadaceae	*P. proteolytica*	99	0.36	0.16
Pseudomonadaceae	*P. fluorescens*	*100*	0.23	< 0.00
Pseudomonadaceae	*P. plecoglossicida**	99	0.08	0.19
Pseudomonadaceae	*P. glareae*	97	0.03	0.02
Pseudomonadaceae	*P. sihuiensis*	*92*	0.01	< 0.00
Pseudomonadaceae	*P. flavescens*	*94*	< 0.00	n/a
Pseudomonadaceae	*P. paralactis*	93	< 0.00	n/a
Pseudomonadaceae	*P. putida*	99	< 0.00	< 0.00
Pseudomonadaceae	*P. qingdaonensis*	93	< 0.00	n/a
Pseudomonadaceae	*P. reidholzensis*	92	< 0.00	< 0.00
Pseudomonadaceae	*P. reinekei*	95	< 0.00	< 0.00
Pseudomonadaceae	*P. turukhanskensis*	97	< 0.00	n/a
Enterobacteriaceae	*Kluyvera cryocrescens*	100	23.01	4.01
Enterobacteriaceae	*Yokenella regensburgei*	97	3.52	0.01
Enterobacteriaceae	*Cedecea lapagei*	93	< 0.00	n/a
Enterobacteriaceae	*Citrobacter braakii*	97	< 0.00	< 0.00
Enterobacteriaceae	*C. werkmanii*	96	< 0.00	n/a
Enterobacteriaceae	*Kosakonia quasisacchari*	95	0.01	n/a
Enterobacteriaceae	*Phytobacter diazotrophicus*	96	< 0.00	n/a
Alcaligenaceae	*Achromobacter deleyi*	100	1.23	0.70
Alcaligenaceae	*A. insolitus*	94	4.43	1.18
Alcaligenaceae	*A. pestifer*	93	0.00	n/a
Alcaligenaceae	*Bordetella avium*	94	0.01	< 0.00

Family	**Species**	**Identity**	**Relative abundance (%)**	
		(%)	**0 mM caff.**	**20 mM caff.**
Xanthomonadaceae	*Stenotrophomonas chelatiphaga*	94	0.26	< 0.00
Xanthomonadaceae	*S. pavanii*	98	0.09	n/a
Xanthomonadaceae	*S. rhizophila*	99	0.01	n/a
Erwiniaceae	*Pantoea cypripedii*	*94*	< 0.00	n/a
Erwiniaceae	*P. wallisii*	*93*	< 0.00	n/a
Yersiniaceae	*Gibbsiella dentisursi*	94	4.69	n/a
Yersiniaceae	*Serratia oryzae*	98	0.02	< 0.00
Brucellaceae	*Brucella pseudogrignonensis*	99	0.01	< 0.00
Flavobacteriaceae	*Flavobacterium acidificum*	99	9.06	0.76
Muribaculaceae	*Muribaculum intestinale*	92	< 0.00	n/a
Pectobacteriaceae	*Pectobacterium atrosepticum*	94	< 0.00	< 0.00
Rhizobiaceae	*Rhizobium nepotum*	*99*	< 0.00	n/a
Sphingobacteriaceae	*Sphingobacterium siyangense*	99	< 0.00	n/a

Note; n/a: No data available, or 0 % of detectability abundant. asterisk (*): Species increased in higher caffeine concentration

Gut bacteria communities cultured in mineral media + 0 mM caffeine

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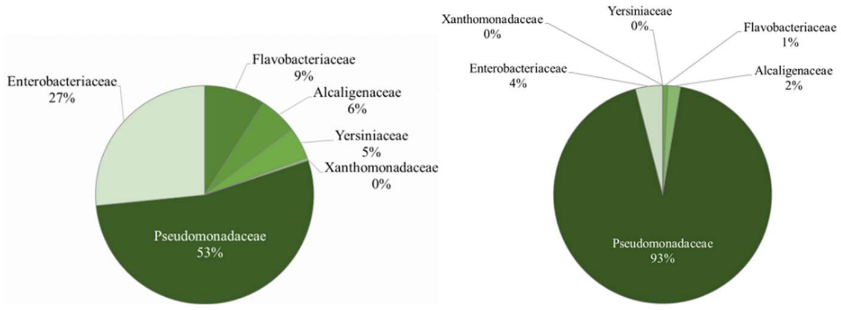
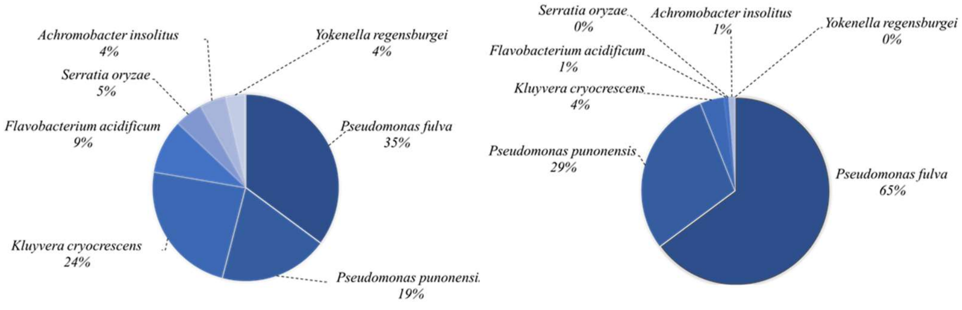
Gut bacteria communities cultured in mineral media + 20 mM caffeine

The bacterial community was significantly decreased in the 20 mM caffeine growth condition. *P. fulva* and *P. punonensis* (family Pseudomonadaceae) were distinctly dominant comprising very high proportions of 64.1 and 28.8 %, respectively (**Figure 3**). Furthermore, *P. plecoglossicida* was found to be increasing in this condition, but only by a small proportion. The overall abundance of the following 17 species decreased in the caffeine growth condition: *P. fluorescens*, *P. glareae*, *P. proteolytica*, *P. reinekei*, *P. sihuiensis*, *Achromobacter deleyi*, *A. insolitus*, *Aerosakkonema funiforme*, *Bordetella avium*, *Brucella pseudogrignonensis*, *Citrobacter braakii*, *Flavobacterium acidificum*, *Gibbsiella dentisursi*, *Kluyvera cryocrescens*, *Pectobacterium atrosepticum*, *Stenotrophomonas rhizophila*, and *Yokenella regensburgei*. Seventeen species of gut bacteria could not survive in the 20 mM caffeine growth condition. This could be attributed to caffeine intolerance of these species. The 17 taxa included *P. flavescens*, *P. paralactis*, *P. qingdaonensis*, *P. turukhanskensis*, *Achromobacter pestifer*, *Cedecea lapagei*, *Citrobacter werkmanii*, *Kosakonia quasisacchari*, *Muribaculum intestinale*, *Pantoea cypripedii*, *P. wallisii*, *Phytobacter diazotrophicus*, *Rhizobium nepotum*, *Serratia oryzae*, *Sphingobacterium siyangense*, *Stenotrophomonas chelatiphaga*, and *S. pavanii*.



Non caffeine condition 20 mM caffeine condition

Figure 2 Community abundance in family level.



Non caffeine condition 20 mM caffeine condition

Figure 3 Community changed in difference of caffeine conditions, showing 7 abundant OTUs.

This experimental study was conducted to assess gut microbiome composition in media with and without caffeine. The potential influence of caffeine on the abundance and diversity of gut bacteria was also assessed. The results of the experiments revealed that the gut microbiome community abundance and species composition significantly changed between the 2 conditions.

Previous studies revealed that the digestive tracts of CBBs contained a wide variety of bacteria [16], which consisted mainly of Pseudomonadaceae, Erwiniaceae, and Brucellaceae [16,9]. In this study, 38 culturable species of gut bacteria were isolated from the caffeine-free media. At the family level, Pseudomonadaceae (53 %) was the dominant one. Enterobacteriaceae (27 %) was the 2nd most dominant, followed by Flavobacteriaceae (9 %), Alcaligenaceae (6 %), and some additional species of the family Xanthomonadaceae, Yersiniaceae, Brucellaceae, Pectobacteriaceae, Erwiniaceae, Muribaculaceae, Oscillatoriaceae, Sphingobacteriaceae and Rhizobiaceae. These results illustrate the diverse community of bacteria present in the gut of CBBs. The community plays crucial role in the insect's ability to use coffee beans as a food source [9], by degrading caffeine and transforming into usable nutrients [1]. Our finding suggested the caffeine degradation potential of several bacteria, such as *P. fulva*, *P. punonensis*, *K. cryocrescens*, *F. acidificum*, *S. oryzae*, and *A. insolitus*. These gut species were also reported previously as caffeine degraders found in the digestive tract of other insects [1,17].

Caffeine is a chemical toxic to insects. This study examined its effect on the gut bacteria community of CBBs. Caffeine intolerant gut bacteria could not grow in the high-concentration caffeine medium of 20 mM. The results of all 6 experiments showed congruent bacteria community changes between non-caffeine and caffeine media. Specifically, 14.5 ± 1.8 OTUs were detected in the non-caffeine media with a significant decrease of 7.2 ± 3.1 OTUs in the caffeine media. Since caffeine eliminates a wide range of bacteria, it can be hypothesized that it can directly affect the chemical defenses and digestion of the insect host. Several of the gut bacteria that could not tolerate coffee, i.e. members of Pseudomonadaceae, Enterobacteriaceae, Xanthomonadaceae, Erwiniaceae, and Yersiniaceae, were reported as important in the metabolic pathways of insects [9]. Specifically, some bacteria produce enzymes that degrade plant materials and cellulose [18,19,8], such as the families Enterobacteriaceae and Erwiniaceae. Both of these were detected in the non-caffeine media, while they were absent in the high caffeine level media in this study. A lack of these bacteria may then make it difficult for insects to digest cellulose and hemicellulose. Fourteen species from Pseudomonadaceae, the caffeine degrading gut bacteria of CBBs [1], were found in the non-caffeine condition, but only 3 of these grew in response to the 20 mM caffeine concentration condition.

Insect pests can be managed by manipulating gut microbiota. CBB gut bacteria have been found to contribute to feeding, parasite and pathogen protection, immune response modulation, and pesticide breakdown [20,2]. Some pesticide degradation bacteria were also found in this study, for example: Organophosphate degradation bacteria (*Pseudomonas* spp., *Pantoea* spp., *Flavobacterium* sp., and *Citrobacter* spp.) and insecticide degradation bacteria (*Stenotrophomonas* spp.). This research revealed 3 species of the family Pseudomonadaceae (*Pseudomonas fulva*, *P. punonensis*, and *P. plecoglossicida*) that preferred to grow in higher caffeine concentrations, while most of the gut bacteria (92 %) responded to caffeine levels by decreasing growth. Therefore, manipulating gut bacteria may lead to successful coffee pest management. More experiments on living CBBs should be conducted in the future to better understand the function of microbiome in pest-insect control. In addition, biopesticides or other plant extracts could be involved in controlling gut bacteria such as *Millingtonia hortensis* L.f. [21], *Paederia pilifera* Hook.f. [22] and *Trema orientalis* (L.) Blume [23]. For sustainable control of CBBs, several methods could be applied by using biological control, e.g., parasitoids control, predators control, entomopathogenic nematodes and entomopathogenic fungi, and integrated pest management, e.g., post-harvest management and traps as an IPM component [24]. This study revealed the caffeine effects on the species diversity of bacteria symbionts in the gut of CBBs hence, providing valuable data on organic coffee plantations in northern Thailand. The results of this study will provide useful data to understand the gut microbiota in CBB and the possible use of coffee insect pest control.

Conclusions

Coffee Berry Borer (CBB) is a significant coffee pest worldwide. Several studies reported the mechanism for caffeine resistance of this coffee pest has evolved with a wide variety of symbiotic gut bacteria. In this study, the effect of caffeine (1,3,7-trimethylxanthine) on CBBs gut bacteria was reported. Caffeine conditions effect on the gut microbiota of CBBs were studied in 2 experimental conditions. Thirty-eight gut bacterial species were isolated from CBBs collected from 6 coffee plantation locations in Pangkhon, Chiang Rai province, north of Thailand. The average number of species and species abundances of gut microbiota were significantly decreased in the 20 mM caffeine condition cultured. In the caffeine condition, the number of gut microbiota taxa decreased by 53 %, with 18 species detected and 20 species absent. The results revealed that the genus *Pseudomonas* (family Pseudomonadaceae) was dominant in the gut of CBBs, and *P. fulva* and *P. punonensis* were distinctly dominant species in caffeine conditions. Our research emphasized the importance of biochemical compound, such as caffeine, conditions on the community of symbiotic bacteria. These might serve as effective obstacles against insect pest outbreaks and sustained coffee insect pest control.

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