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Contributed Paper

Gut Bacterial Diversity in *Plasmodium*-infected and *Plasmodium*-uninfected *Anopheles minimus*

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ABSTRACT

Anopheles minimus is one of the main malaria vectors in Thailand. *Plasmodium* transmission depends primarily on the success of the parasite survival in the mosquito's gut. Several factors affect the development of *Plasmodium* in the mosquito, including the gut microbiota. Here, we used culture-independent method to identify microbiota and compared the bacterial communities in the gut of *Plasmodium*-infected and *Plasmodium*-uninfected mosquitoes. Fifty-three genera within four phyla were detected and 14 of them were discovered in malaria vectors for the first time. In addition, we found that the bacterial diversity and the profile of the gut bacterial communities between the *Plasmodium*-infected and those of the uninfected mosquitoes were quite different. The result showed that the bacterial diversity in the gut of the uninfected mosquitoes was also much higher than that of the infected counterpart. Gammaproteobacteria were prevalent in the infected *An. minimus* while betaproteobacteria were the most abundant in the uninfected mosquitoes. Three genera, *Acinetobacter* in gammaproteobacteria, *Alcaligenes* and *Burkholderia* in betaproteobacteria were the core set of bacteria found in the gut of the malaria vector.

Keywords: *Anopheles minimus*, gut microbiome, 454 sequencing, malaria vector, *Plasmodium*

1. INTRODUCTION

Malaria is one of the serious public health concerns in several countries. Approximately 3.4 billion people worldwide are at risk of being infected with malaria [1]. In 2012, there were estimated 207 million cases of malaria and 627,000 deaths [1]. Although the disease can be cured by anti-malarial drugs, the resistance of *Plasmodium* to the medicines has been found worldwide, especially in Asia [2,3]. Even though using of bed net and indoor

insecticides has been widely practiced, a number of people are still infected with *Plasmodium*. Hence, the alternative protection methods have been developed such as symbiotic control, which is the use of natural symbiotic microorganisms to control vector-borne diseases [4,5].

Plasmodium transmission depends primarily on the success of the parasite survival in the mosquito gut. The lumen is the first place *Plasmodium* will attach, grow and transform to the next developmental stages in the life cycle [6]. However, a number of the parasites were dramatically reduced in the gut phase [6]. Many factors that affect *Plasmodium* development in *Anopheles* mosquitoes including microbiota in the anopheline midgut have been reported [6,7]. For two decades, researchers have tried to isolate bacteria in the gut of *Anopheles* species and studied the relationship between these bacteria and *Plasmodium* development [5]. They also found that certain bacterial strains could inhibit the growth of *Plasmodium* [7,8]. Since only a small number of microorganisms could grow in synthetic media, culture-independent methods, which incorporate the use of next generation sequencing (NGS) technology, have been applied to investigate unculturable microbial communities. In the past few years, NGS has widely been used to study microbial diversities from various sources, including *Anopheles* mosquito gut, especially *An. gambiae* [9,10,11]. However, identification of gut microbiome in *An. minimus*, a malaria vector, has not been carried out using NGS technology.

An. minimus is one of the main malaria vectors in Thailand, found primarily in forest regions along the border. In our work, bacterial diversity in the gut of *An. minimus,* and bacterial communities of *Plasmodium*-infected and *Plasmodium*-uninfected mosquitoes were identified and compared using culture-independent method.

2. MATERIALS AND METHODS

2.1 Mosquito Collection and Ethics Statement

Mosquitoes were collected at Mae Sot district, Tak Province, Thailand using outdoor human-landing collections. This site is located close to a refugee camp on Thai-Myanmar border and it is one of the malaria-endemic regions in Thailand. All specimens were kept in plastic cups, which contained cotton soaked with 10% sterile sugar solution and stored at -80°C until use. The mosquitoes were surface rinsed with 70% ethanol. Head-thorax and gut sections were dissected and used for species identification and bacterial identification, respectively.

Formal animal/human use approval for this research was granted by the Ethic Review Committee for Research Involving Human Research Subject, Health Science Group, Chulalongkorn University (COA No. 167.2013).

2.2 Species Identification and Detection of Plasmodium Infection

DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instruction and then it was used for species identification in *An. minimus* complex according to the method of Sharpe *et al.* [12]. The result showed that the mosquitoes were *An. minimus*.

Detection of *Plasmodium* infection followed the method of Rougemont *et al.* [13]. PCRs were performed in a final volume of 20 µL consisting of 2 µL of DNA template, 1 U of GoTaq polymerase (Promega, USA), 1x *Taq* reaction buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 500 nM of each primer and sterile distilled water to make up the remainder of the 20–µL volume.

Conditions used for amplification in a thermocycler (Biometra, Germany) were as follows: pre-incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min and a final extension step at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel, purified using the QIAquick Gel Extraction kit (Qiagen, Germany) and sequenced by Macrogen (Korea). The local alignment results indicated that certain specimens were infected with *Plasmodium falciparum*. Six guts from three *Plasmodium*-infected and three *Plasmodium*-uninfected female mosquitoes were used for 16S rRNA amplicon survey study.

2.3 Gut Bacterial Species Identification

DNA isolated from the gut was used as a template in PCR reactions. Additional eight-nucleotide sequences [14] (Table 1) attached to two primers; 347F (5'-GGAG GCAGCAGTRR-GGAAT-3') and 803R (5'-CTACCRGGGTATCTAATCC-3') [15], were used to tag each mosquito's gut specimen. The partial 16S rRNA gene was amplified using HotStar Hifidelity Polymerase kit (Qiagen, Germany). The reaction contained 2 µL of DNA template, 1 U of HotStar Hifidelity Polymerase (Qiagen, Germany), 1x HotStar Hifidelity PCR buffer containing 1.5 mM $MgSO_4$ and 0.3 mM dNTPs, 500 nM of each primer and sterile distilled water to make up the remainder of the 20-µL volume. The amplification cycles were as followed: pre-incubation at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min and a final extension step at 72°C for 5 min. The PCR products were then purified with MinElute PCR Purification kit (Qiagen, Germany) and their concentrations were measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). The integrity of the DNA was verified by a Bioanalyzer (Agilent) prior to sequencing. Purified PCR products from each mosquito were diluted to the same concentration and pooled in equimolar amount. Approximately 200 ng of pooled DNA was sequenced using a GS-FLX Titanium platform (Roche Applied Science, Germany). The sequencing was carried out according to the manufacturer's protocol.

Table 1. Nucleotide sequences of primers and tags used in the present study. The sequence of each tag was underline.

Primer name	Sequence (52 ->32)
347F-01	TCTCTGTGGGAGGCAGCAGTRRGGAAT
803R-01	TCTCTGTGCTACCRGGGTATCTAATCC
347F-02	TCTACTCGGGAG GCAGCAGTRRGGAAT
803R-02	TCTACTCGCTACCRGGGTATCTAATCC
347F-03	TAGTAGCGGGAGGCAGCAGTRRGGAAT
803R-03	TAGTAGCGCTACCRGGGTATCTAATCC
347F-04	AGACGACGGGAGGCAGCAGTR RGGAAT
803R-04	AGACGACGCTACCRGGGTATCTAATCC
347F-05	ACTCGTAGGGAGGCAGCAGTRRGGAAT
803R-05	ACTCGTAGCTACCRGGGTATCTAATCC
347F-06	ACATCGAGGGAGGCAGCAGTRRGGAAT
803R-06	ACATCGAGCTACCRGGGTATCTAATCC

2.4 Sequence Cleaning

Sequences obtained from the GS-FLX Titanium sequencer were demultiplexed according to the tagged barcode sequences. The sequences were cleaned by trimming the 454 adapter and barcodes using the custom python script. Chimeric sequences were identified and removed using UCHIME [16] against referenced database from SILVA [17]. To improve the robustness of analyses, the clean reads were then filtered and size-selected: only high-quality reads that were at least 200 nucleotides in length were included in further analyses. The 16S rRNA libraries sequences of bacteria from the guts are available in the Sequence Read Archive (SRA) on NCBI with the accession number SRX481169.

2.5 Taxonomic Classification and Statistical Analysis of Pyrosequencing Data

The cleaned sequences from the previous step were assigned their phylotypes using Ribosomal Database Project (RDP) naive Bayesian Classifier [18] with 80% confidence threshold. Operational taxonomic units (OTUs) were determined at sequence similarity levels of 85%, 90%, 95%, and 97% by MOTHUR [19] based on the furthest-neighbor method. The richness (sobs), the Chao1 richness estimator, the abundance-based coverage estimator (ACE) and the Shannon-Weaver diversity index were calculated using MOTHUR software in order to compare microbial diversity between the *Plasmodium*-infected and the *Plasmodium*-uninfected mosquitoes. Good's coverage was calculated as $G = 1 - n/N$, where n is the number of singleton phylotypes and N is the total number of sequences in the sample. The visualization and comparison of microbial communities were performed by STAMP (Statistical Analysis of Metagenomic Profiles) [20] .

3. RESULTS

3.1 454 Sequencing and Statistical Analyses

We obtained a total of 42,599 raw reads with the average read length of 272 nucleotides. After the adapter and low-quality trimming, 25, 641 filtered reads (with the mean read length of 360 bases) were assigned the phylotypes using the RDP classifier (Table 2). We were able to designate 99.96% of the reads as originating from bacteria, and 92.30% as well as 83.32% of the trimmed reads were assigned to bacteria at the family and the genus levels, respectively (Table 2). To analyze whether the diversity of the gut microbiome is sufficiently covered by our sequence data, rarefaction and species richness calculations were performed. We carried out the rarefaction analyses at four different dissimilarity cutoffs. At sequence similarity levels of 85% and 90%, the rarefaction curves computed for three *Plasmodium*-infected and three uninfected specimens appeared to have leveled off, suggesting that our sequence data have covered almost all phylogenetic groups underlying the gut microbial communities at the family/class levels (Figure 1). At sequence similarity levels of 95% and 97%, the dataset did not seem to reach the plateau, however, the percentages of Good's coverage at all taxonomic levels were very high (Table 3). We also applied statistical models to assess the bacterial diversity of the *An. minimus* gut metagenomes (Table 3).

At all sequence similarity levels, the richness of the uninfected mosquitoes was 1.30-1.64 folds higher than that of the infected ones. The Chao1 and ACE values of the uninfected ones were higher than those of the infected mosquitoes at 1.62-1.85 and 1.60-2.12 fold, respectively. However, the values of Shannon index of the uninfected and the infected specimens were not different

(0.99-1.02). Altogether, the metrics suggested that the gut microbiome of the uninfected mosquitoes display a slightly higher degree of diversity compared to the *Plasmodium*-infected individuals.

Table 2. Summary of 16S rRNA tagged-pyrosequencing data from three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

Sample	Raw sequence		Cleaned sequence	
	Number of sequence	Average length (bp)	Number of sequence	Average length (bp)
PI1	5,041	284.21	3,350	356.59
PI2	1,629	222.03	694	387.34
PI3	10,106	323.68	7,851	370.82
PU1	12,867	229.05	5,833	347.40
PU2	6,355	272.57	3,881	353.76
PU3	6,601	275.94	4,032	359.88
Total	42,599	263.51	25,641	359.78

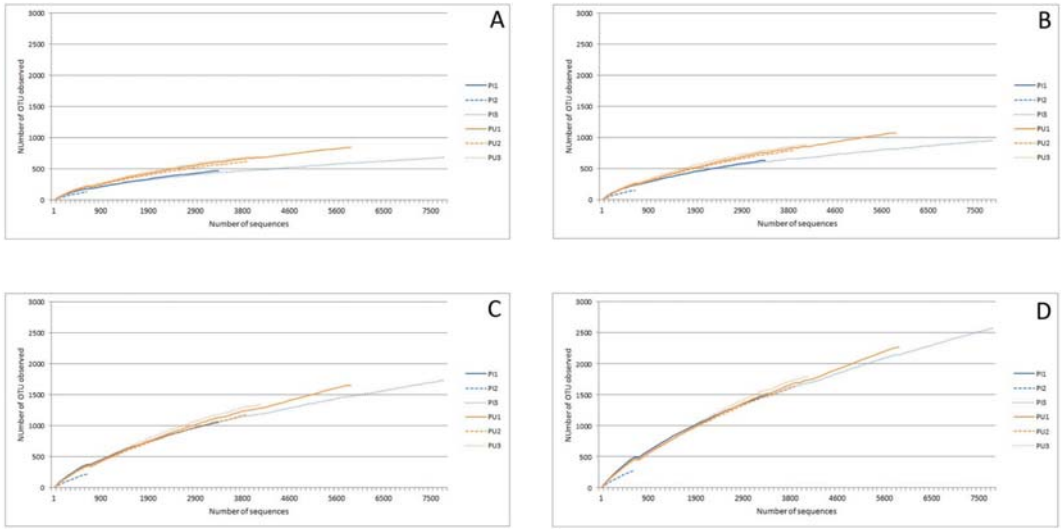

Figure 1. Rarefraction curves of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus* at four similarity levels; 85% (A), 90% (B), 95% (C) and 97% (D), respectively.

Table 3. Statistical analysis and biodiversity index of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus* at similarity levels of 85%, 90%, 95% and 97 %.

Sample	Similarity level	Richness	Chao1	ACE	Shannon	Good coverage (%)
PI	85%	1,004	1,806	2,472	4.7064	95.76
PI1		470	951	1,489	4.3999	92.24
PI2		130	241	355	2.9468	89.19
PI3		691	1,327	1,737	4.4769	95.54
PU		1,647	3,282	4,849	4.8233	93.37
PU1		849	1,763	2,476	4.3774	91.67
PU2		623	1,423	1,985	4.1966	90.60
PU3		703	1,386	2,008	4.6006	90.20
PI	90%	1,379	2,517	3,523	5.3125	94.05
PI1		634	1,232	2,085	4.9437	89.40
PI2		153	351	586	3.1219	85.88
PI3		955	1,773	2,388	5.1161	93.90
PU		2,150	4,649	7,473	5.3186	90.77
PU1		1,078	2,463	3,987	4.8561	88.69
PU2		798	1,929	2,981	4.6932	87.19
PU3		888	1,923	2,970	4.9427	86.83
PI	95%	2,472	5,255	7,912	6.4946	88.05
PI1		1,059	2,317	3,599	6.1181	80.99
PI2		222	661	911	3.7611	76.95
PI3		1,745	3,546	5,014	6.2664	87.62
PU		3,402	8,931	15,281	6.4340	83.66
PU1		1,657	4,430	7,822	5.8876	80.80
PU2		1,185	2,997	5,208	5.7522	79.82
PU3		1,346	3,689	6,059	5.8430	77.21
PI	97%	3,651	8,637	14,622	7.2968	80.82
PI1		1,485	3,544	6,182	6.8210	71.31
PI2		282	861	2,177	4.3715	69.31
PI3		2,588	6,010	9,3222	7.0339	79.51
PU		4,734	13,984	3,449	7.4156	76.27
PU1		2,269	7,355	13,729	6.7847	72.00
PU2		1,629	4,305	7,622	6.5854	71.09
PU3		1,798	5,225	9,032	6.7272	68.18

3.2 Gut Bacterial Classification

Taxonomic classification with the RDP Classifier detected the presence of 53 genera in four bacterial phyla in the gut of *An. minimus* (Table S1). Proteobacteria was far more abundant than the other groups, representing 95.02% of the OTUs assigned and containing the 43 distinct genera (Table 4). At the class level, the gut community was dominated by two taxonomic classes: gammaproteobacteria

(57.40%) and betaproteobacteria (35.12%; Table S1). Among members of the gammaproteobacteria, *Moraxellaceae* was associated with 28.79% of the sequences while other predominant OTUs were assigned to *Enterobacteriaceae* (16.50%). For betaproteobacteria, the major bacterial groups were *Alcaligenaceae* (16.26%) and *Burkholderiaceae* (15.79%). The variation of the gut bacterial communities among the specimens at phylum and class levels could be observed, however, composition patterns of gut microbiota of *An. minimus* in the same group were quite similar (Figure 2). At the genus level, only twenty six groups of bacteria represented more than 1% of the total OUTs in each specimen are shown in Figure 3. Most of them were detected in at least 50% of all specimens examined. The result showed that the most abundant genera (30% of all OTUs in at least one specimen) were *Alcaligenes, Burkhoderia*, *Thorsellia*, unclassified *Enterobacteriaceae* and *Acinetobacter* (Figure 3).

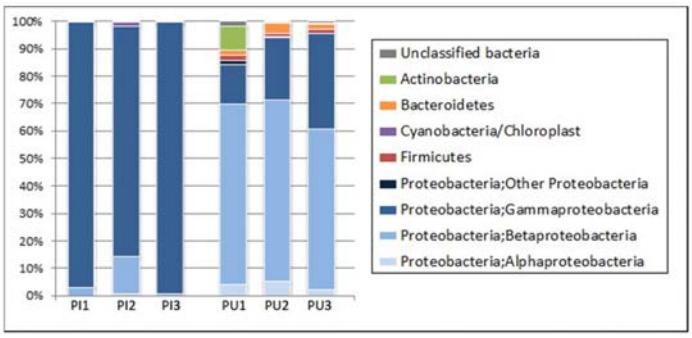

Figure 2. Taxonomic classification of microbiota in the gut of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

Figure 3. Heat map of bacterial profiles in the gut of the *Plasmodium*-infected (PI) and the *Plasmodium*-uninfected *An. minimus*. Only genera represented more than 1% of the total OTUs in each specimen were included.

3.3 Bacterial Community in *Plasmodium*-infected and *Plasmodium*-uninfected Hosts

Out of 53 genera, 20 were found in both infected and uninfected mosquitoes while 7 and 26 genera were detected only in the *Plasmodium*-infected or the *Plasmodium*-uninfected individuals, respectively (Table 4). The majority of bacteria presented in the gut of the infected host were gammaproteobacteria (97.40%), followed by betaproteobacteria (2.19%; Figure 4). *Moraxellaceae* (69.50%) and *Enterobacteriaceae* (26.28%) families were the most abundance in the gammaproteobacteria (Figure 4). The scatter plot showed that *Acinetobacter* in the *Moraxellaceae*, *Thorsellia* in the *Enterobacteriaceae* and unclassified *Enterobacteriaceae* were abundant in the *Plasmodium*-infected hosts (Figure 5). In contrast, the gut of the uninfected mosquitoes was dominated with betaproteobacteria (63.62%; Figure 4). Moreover, Actinobacteria, alphaproteobacteria, Firmicutes and Bacteroidetes were almost exclusively found in the gut of the uninfected mosquitoes. Among them, *Burkholderia* in the family *Burkholderiaceae*, *Alcaligenes* in the family *Alcaligeneceae* and *Serratia* in the family *Enterobacteraceae* were the most prevalent (Figure 4).

Table 4. The 53 genera discovered in the gut of *Anopheles minimus*. Certain bacterial genera were detected in both *Plasmodium*-uninfected and infected mosquitoes (B) whereas some of them were presented only in the *Plasmodium*-uninfected mosquito (PU) or in the *Plasmodium*-infected mosquito (PI).

Phylum/class	Genus	Detection (B, PU, PI)	Percentage from total OTUs
Actinobacteria	Corynebacterium[a]	PU	0.07
	Brachybacterium[a,b]	B	0.47
	Kocuria[a]	PU	0.07
	Micrococcus[a]	PU	1.22
	Propionibacterium[a]	PU	0.16
Bacteroidetes	Elizabethkingia[a]	PU	1.05
	Flavobacterium[a]	B	0.02
	Flavisolibacter[a,b]	PU	0.12
Firmicutes	Bacillus[a]	PU	0.03
	Staphylococcus[a]	B	0.71
Proteobacteria/ Alphaproteobacteria	Brevundimonas[a]	PU	0.02
	Bradyrhizobium[a]	PU	0.01
	Nitrobacter[a,b]	PU	0.04
	Methylobacterium[a]	B	0.08
	Mesorhizobium[a,b]	PU	0.04
	Rhizobium[a,b]	PU	0.10
	Asaia[a]	PU	0.01
	Roseomonas[a]	PU	0.13
	Novosphingobium[a]	B	0.13
	Sphingomonas[a]	B	0.61

Table 4. Continued.

Phylum/class	Genus	Detection (B, PU, PI)	Percentage from total OTUs
Proteobacteria/ Betaproteobacteria	*Achromobacter*[a]	B	2.48
	Alcaligenes[a]	PU	14.69
	Burkholderia[a]	B	14.59
	Ralstonia[a]	PU	0.06
	Aquabacterium[a]	PU	0.04
	Acidovorax[a]	PU	0.08
	Comamonas[a]	B	0.01
	Delftia[a]	B	0.51
	Tepidicella[a,b]	PI	<0.01
	Variovorax[a,b]	PU	<0.01
	Massilia[a,b]	B	0.31
	Naxibacter[a,b]	PU	<0.01
	Methyloversatilisa[a,b]	PU	0.01
Proteobacteria/ Deltaproteobacteria	*Desulfohalobium*[a,b]	PU	0.23
Proteobacteria/ Gammaproteobacteria	*Aeromonas*	PI	<0.01
	Pseudoalteromonas[a,b]	PU	0.37
	Citrobacter	PI	<0.01
	Enterobacter	PI	<0.01
	Escherichia/Shigella	PI	0.02
	Klebsiella[a]	B	0.41
	Pantoea	PI	<0.01
	Salmonella[a]	B	0.16
	Serratia	B	3.94
	Thorsellia[a]	B	2.08
	Trabulsiella[a,b]	PI	<0.01
	Zymobacter[a]	PU	0.02
	Acinetobacter	B	32.37
	Enhydrobacter[a]	B	1.35
	Pseudomonas	B	1.20
	Lucibacterium[a,b]	PU	0.09
	Vibrio[a]	PU	0.02
	Stenotrophomonas[a]	B	2.32
	Xanthomonas[a,b]	B	1.37

Note: [a] the new genera firstly detected in *Anopheles minimus*.

[b] the new genera detected in *Anopheles* species for the first time.

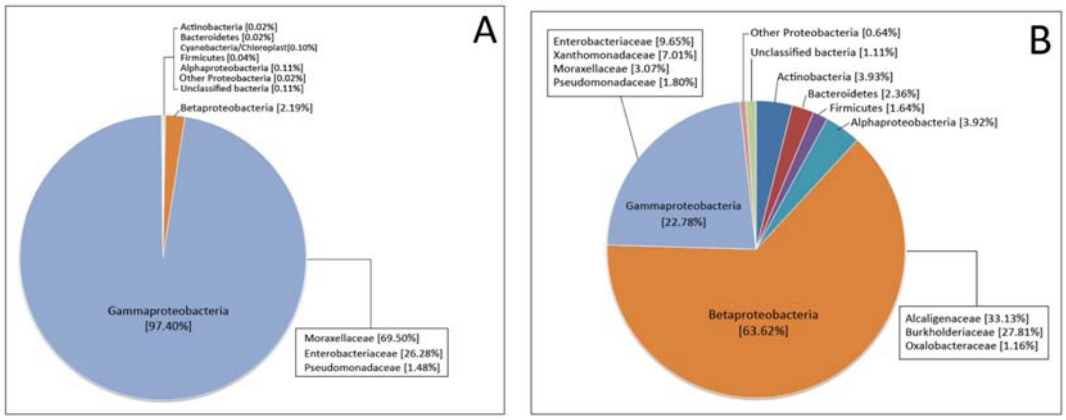

Figure 4. Percentages of relative abundance at taxonomic levels. (A) and (B) indicate the relative abundance (%) of bacteria in the gut of the *Plasmodium*-infected and the *Plasmodium*-uninfected *An. minimus*, respectively.

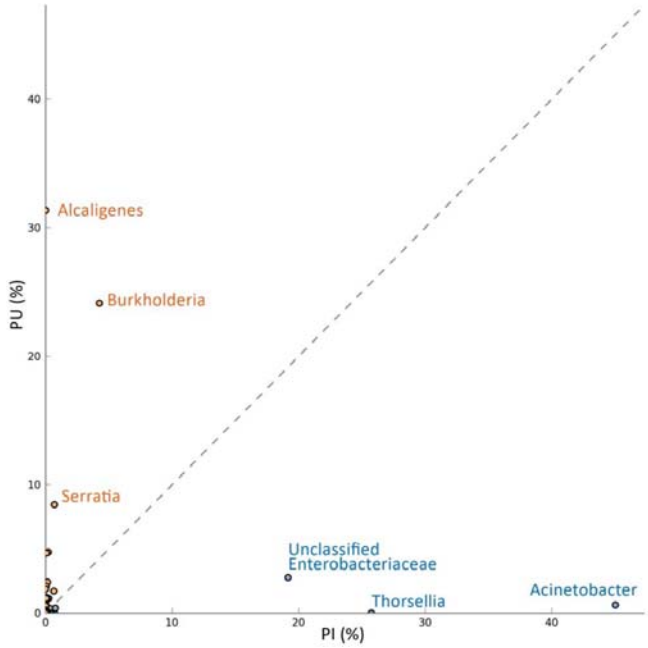

Figure 5. Scatter plot of prevalent bacterial genera comparing between the gut microbiota in the *Plasmodium*-infected (PI; PI1, PI2, PI3) and the *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

4. DISCUSSION

4.1 First Discovery of Certain Bacterial Genera in the Gut of *An. minimus*

In the present study, 45 of the total 53 genera were detected for the first time in *An. minimus*. Of the 45 genera, 31 were found in other malaria-vectors while one genus, *Enhydrobacter*, was present in a non-malaria vector, *An. barbumbrosus* [11, 21]. Six of ten midgut core microbiota, *Burkholderia, Serratia, Acinetobacter, Pseudomonas, Sphingomonas* and *Staphylococcus* detected in *An. gambiae* in a

previous study were also abundant in the current study [11]. Similarly to previous reports, a small proportion of gram-positive bacteria, Actinobacteria and Bacilli, was found in the gut of our specimens [10, 11]. However, 14 genera discovered in the present study have never been found in any *Anopheles* species. Most of them were present in low frequency with an exception of *Xanthomonas*, the abundance of which was higher than 1%. All 14 genera were isolated from plant tissues, soils, air and water resources [22]. These findings suggested that the mosquitoes might acquire these bacteria by feeding on plant sap or becoming in contact with the environments that contained the bacteria.

4.2 The Relationship between Gut Bacteria and *Plasmodium*

A large number of microorganisms are generally present within the body of mosquito, especially in the gut [6]. Several studies have reported the benefits of gut bacteria to the host, including parasite-development inhibition [23]. Antibiotic-treated *An. gambiae* and *An. stephensi* had lower bacteria contents and higher *P. falciparum* infection rates than the untreated mosquitoes [24]. In addition, intrathoracic inoculation of bacteria in *An. gambiae* induced antibacterial peptide production that inhibited *Plasmodium* development [25]. Moreover, Cirimotich *et al.* [7] revealed that a strain of *Enterobacter* isolated from wild-caught *An. arabiensis* could kill *P. falciparum* using reactive oxygen species . Recently, *Serratia marcescens* HB3 had been shown to inhibit *P. berghei* oocyst formation in *An. stephensi* [23]. These findings suggested that certain gram-negative bacteria play an important role to protect mosquitoes from *Plasmodium* infection.

The gram-negative bacteria, *Acinetobacter*, were the most abundant bacteria in the gut samples from our study. It was found that *Acinetobacter* species isolated from wild-caught *An. arabiensis* could reduce the number of oocysts in the midgut of *An. gambiae* via the activation of the immune deficiency (IMD) immune signaling pathway [26]. In addition, the infection of *Acinetobacter* in the mosquito midgut reduced longevity of *An. gambiae* [26]. *Elizabethkingia meningoseptica* and *Serratia marcescens* were also detected in our work (data not shown) and they previously exhibited anti-*Plasmodium* activity in *Anopheles* species [8, 23, 26]. *E. meningoseptica* was able to inhibit the development of *P. falciparum* at the gametocyte transmission stage [27]. Recently, Bahia *et al.* [26] reported that the anti-*Plasmodium* activity of *S. marcescens* derived from secreted factors. The findings demonstrated that *Acinetobacter*, *S. marcescens* and *E. meningoseptica* could be used for malarial control in *Anopheles* mosquitoes including *An. minimus*.

4.3 Core Gut Microbiota and Symbiotic Candidates for Malaria Control

Even with regular applications of insecticides and the availability of antimalarial drugs, over 200 million of malaria cases have been reported every year [28]. Unfortunately, malaria vectors have exhibited increased resistance to the insecticides and the antimalarial drugs has become less effective [28]. Novel preventive strategies including symbiotic control might be an alternative to other conventional approaches. The symbiotic control is a strategy to control insect borne diseases by reducing vector competence [4]. A symbiotic candidate should be selected from core gut microbiota, which is abundant and mainly found in a host species. In our study, there were 20 genera residing in the gut of the mosquitoes higher than one percent of all OTUs in each specimen; however only three genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* represented more than 10% of the

total OTUs. According to the abundance of the three genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* were core gut microbiota in *An. minimus* and were eligible symbiotic candidates for malaria control. *Acinetobacter* have the ability to reduce the number of *Plasmodium* oocysts in the midgut of *An. gambiae* [26], so we could use them directly to control the transmission of malaria in the malaria-vector. In contrast, the anti-*Plasmodium* activity in *Alcaligenes* and *Burkholderia* has not been reported. *Burkholderia* appeared to colonize in the gut of all mosquitoes examined. This genus is known as an insect symbiont, and it increases bacterial and fungal resistance of insects [29]. *Alcaligenes* was the most prevalent in the gut of the uninfected *An. minimus,* and it was detected in all of the uninfected samples. *Alcaligenes* has been used as a symbiont to control insect borne diseases in the plants [30]. However, the relationship between the two genera, *Burkholderia* as well as *Alcaligenes*, and *Plasmodium* is still unclear. Further studies are required to determine whether these bacteria possess any activities that lead to the inhibition of *Plasmodium* development in the mosquito gut. Certain species of bacterial flora were shown to trigger mosquito innate immune responses against *Plasmodium* infection [27].

5. CONCLUSION

Bacterial communities in the gut of the infected and the uninfected *Anopheles minimus* were different. In the uninfected mosquito, the gut bacteria were more diverse than those in the infected mosquito. Many bacterial genera in this study were detected for the first time in malaria vectors but they were present only in a small proportion, except *Xanthomonas*. Our study showed that three bacterial genera, *Acinetobacter*, *Burkholderia* and *Alcaligenes* were the most abundant. One of them, *Acinetobacter* has been reported that it had the ability to control the *Plasmodium* transmission by activating the IMD immune response of *Anopheles* mosquito whereas the relationship between the other two bacteria and *Plasmodium* are unclear.

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