Final Report

Project Title: Isolation and identification of microbiota in the midgut of a malaria vector, *Anopheles dirus* complex

By Wunrada Surat

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Abstract

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Project Period: 2 years

มาลาเรียมีสาเหตุมาจากการติดเชื้อพลาสโมเดียมซึ่งมียุงก้นปล่องในสกุล *Anopheles* เป็นพาหะนำโรค การแพร่ระบาดของโรคขึ้นกับหลายปัจจัย ซึ่งรวมถึงแบคทีเรียที่อาศัยในทางเดินอาหารส่วนกลางของยุงก้นปล่อง ในงานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจสอบชนิดของแบคทีเรียที่อาศัยอยู่ในทางเดินอาหารของยุงก้นปล่อง 2 กลุ่ม คือ *Anopheles dirus* complex กับ *An. minimus* ซึ่งเป็นพาหะหลักที่พบในประเทศไทย จากการจำแนกเชื้อแบคทีเรียด้วยวิธีการแยกเชื้อบนอาหารสังเคราะห์ พบแบคทีเรีย 2 สกุล คือ *Bacillus* species และ *Staphylococcus* species ในทางเดินอาหารของ *An. scanloni* (*An. dirus* C) และพบแบคทีเรีย 5 สกุล คือ *Chryseobacterium, Enterobacter, Acinetobacter, Paenibacillus* และ *Cellulosimicrobium* ในทางเดินอาหารของยุง *An. minimus* แต่เมื่อตรวจสอบความหลากหลายของแบคทีเรียด้วยวิธี metagenomics สามารถตรวจสอบได้เฉพาะแบคทีเรียในทางเดินอาหารของยุง *An. minimus* เท่านั้น และพบแบคทีเรียทั้งหมด 61 สกุล ในจำนวนนี้มี 3 สกุลที่อาศัยอยู่ในทางเดินอาหารของยุงมากกว่า 10 % ของแบคทีเรียที่พบทั้งหมด ได้แก่ *Acinetobacter, Burkholderia* และ *Alcaligenes* แสดงให้เห็นว่าแบคทีเรียกลุ่มนี้สามารถเจริญได้ดีในทางเดินอาหารของยุง *An. minimus* ดังนั้น เราสามารถนำแบคทีเรียเหล่านี้ไปใช้ในการควบคุมการแพร่ระบาดของมาลาเรียได้ด้วยวิธี symbiotic control

คำสำคัญ: พาหะนำโรคมาลาเรีย ทางเดินอาหารส่วนกลาง การศึกษาด้วยวิธี metagenomics พลาสโมเดียม

Malaria is caused by *Plasmodium* infection which mosquitoes in the genus *Anopheles* are malaria vectors. Many factors affect the transmission of malaria including bacteria in the midgut of the mosquitoes. In this research, we aimed to explore the bacterial diversity in the midgut of *Anopheles dirus* complex and *An. minimus* which are the main-malaria vectors in Thailand. The results of the culture-dependent method showed that the midgut of *An. scanloni* contained *Bacillus* species and *Staphylococcus* species. In the midgut of *An. minimus*, we found 5 bacterial genera; *Chryseobacterium, Enterobacter, Acinetobacter, Paenibacillus* and *Cellulosimicrobium*. For metagenomic study, only the amplification of *An. minimus* samples has been successful. A total of 61 genera were discovered. Of the 61, only 3 genera*, Acinetobacter, Burkholderia* and

Alcaligenes, were abundance with the percentage higher than 10%. This revealed that these 3 bacterial genera can grow and adapt well in the midgut of *An. minimus*. Furthermore, they are symbiotic candidates used for malaria control.

Keywords : malaria vectors, midgut, metagenomic study, Plasmodium

2. Executive summary

Here, we showed midgut bacterial diversity using the culture-dependent and the culture-independent methods. Using the culture-dependent method, *Staphylococus* and *Bacillus species* were found in the midgut of *An. scanloni*, while *Chryseobacterium, Enterobacter, Acinetobacter, Paenibacillus* and *Cellulosimicrobium* were detected in the midgut of *An. minimus*. Using the culture-independent method, 61 genera were identified. Interestingly, many of them, such as *Acinetobacter, Elizabethkingia* and *Serratia*, were reported that they could directly and indirectly inhibit Plasmodium development. These bacteria are possibly used as malaria control. In addition, the abundance of *Bulkholderia* and *Alcaligenes* in the midgut of *An. minimus* suggested that they have the potential for paratransgenesis.

3. Objective

To identify bacterial diversity in the midgut of *Plasmodium*-infected and uninfected *An. minimus* and *Anopheles dirus* mosquitoes using both culture-dependent and culture-independent methods.

4. Research methodology

Mosquito collection and ethics statement

Mosquitoes were collected from Tak and Satul Provinces using outdoor human-landing collections. All samples were kept in plastic cups, which contained cotton soaked with 10% sugar solution and stored at -80^{o}C until use. The mosquitoes were surface rinsed with 70% ethanol. Abdomen and head-thorax were dissected with a sterilized scalpel. The head-thorax sections were used for species identification whereas the abdomens were used for bacterial identification.

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).

Species identification and detection of plasmodium infection

DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instruction. One microliter of DNA was used for species identification in *An. minimus* complex and *An. dirus* complex according to the method of Sharpe et al. (1999) as

well as Boonkue and Arunyawat (2013), respectively. The results showed that the mosquitoes were *Anopheles minimus* and *An. scanloni*.

Detection of Plasmodium infection was followed by the method of Rougemont et al. (2004). The local alignment results indicated that certain samples were infected with *Plasmodium falciparum*. Six midguts from three Plasmodium-infected and three Plasmodium-uninfected female mosquitoes were used for metagenomic study.

Midgut bacterial species identification:

Culture-dependent method: A total of 15 and 10 Plasmodium-free *An. minimus* and *An. scanloni* were used for the identification of bacteria residing in the midgut. Isolation of midgut bacteria was followed the method of Rani et al. (2009). Each isolate was species-identified by PCR technique using universal 16S rRNA primers (Weisburg et al. 1991). PCR products were sequenced by Macrogen (Korea) and DNA sequences were analyzed using Blast program.

Culture-independent method: DNA isolated from six abdomens of *An. minimus* (three infected and three uninfected mosquitoes) was used as a template in PCR reactions. Additional eight-nucleotide sequences (Humblot and Guyot 2009; Table S1) attached to two primers; 347F (5'-GGAGGCAGCAGTRRGGAAT-3') and 803R (5'-CTACCRGGGTATCTAATCC-3'; Nossa et al. 2010), were used to tag each mosquito's midgut sample. The partial 16S rRNA gene was amplified using HotStar Hifidelity Polymerase kit (Qiagen, Germany). 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.

Sequence analysis

The sequences were cleaned by trimming the 454 adapter and tagged sequences using the custom python script. Chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011). Only high-quality reads that are at least 100 nucleotides in length were included in further analyses. The whole metagenome sequences of bacteria from the midguts are available in the Sequence Read Archive (SRA) on NCBI with the accession number SRX481169.

Taxonomic classification and statistical analysis of pyrosequencing data

The cleaned sequences from the previous step were assigned their phylotypes using RDP classifier (Wang et al. 2007) with 80% confidence threshold and BLASTN against NCBI 16S microbial database. Operational taxonomic units (OTUs) were determined at sequence dissimilarity levels of 0.03, 0.05, and 0.15 by MOTHUR (Schloss et al. 2009) based on the

furthest-neighbor method. The Shannon-Weaver diversity index, the Chao1 richness estimator, and the abundance-based coverage estimator (ACE) were calculated using MOTHUR software in order to compare microbial diversity between infected and non-infected 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) (Parks and Beiko 2010).

5. Result

DNA extraction and identification of mosquitoes

DNA of 15 *An. minimus* and 10 *An. dirus* complex was successfully extracted. Then, to identify species and strain of these mosquitoes, The DNA samples were used as templates in PCR reaction. The results showed that all 15 *An. minimus* complex are *An. minimus* (previously called *An. minimus* A) (Fig. 1), while all 10 *An. dirus* complex are *An. scanloni* (previously called *An. dirus* C) (Fig. 2).

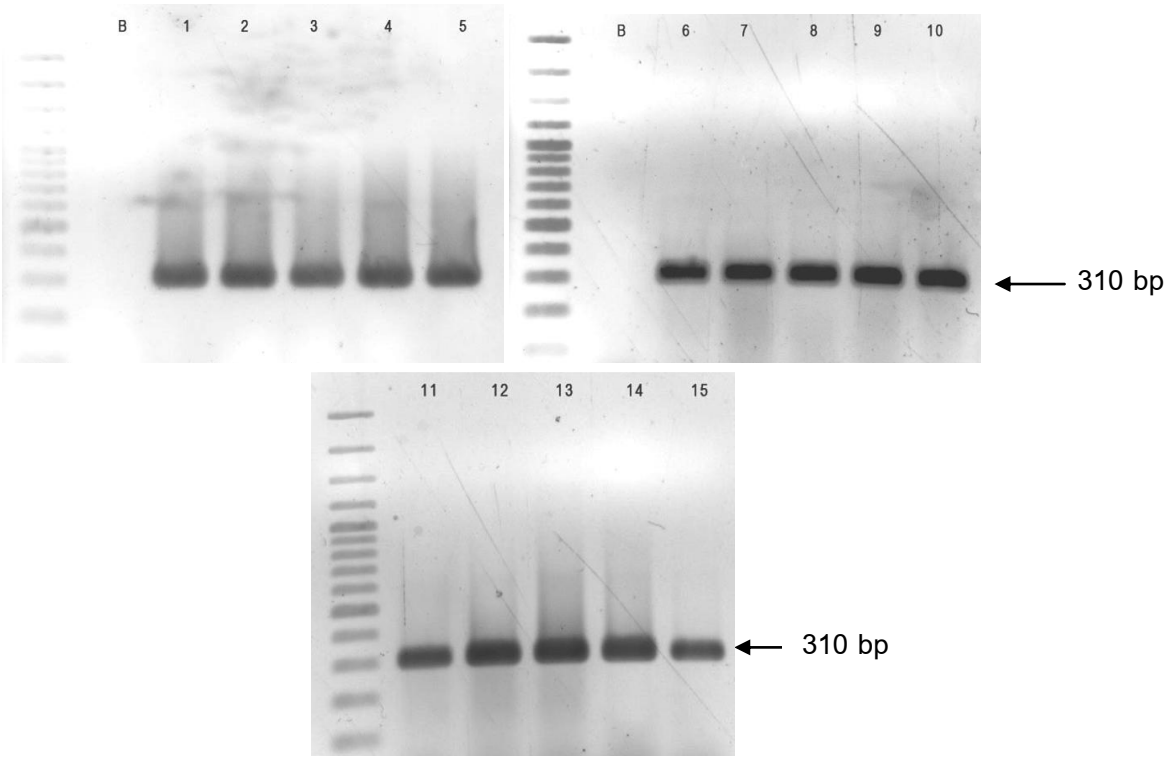

Figure 1 PCR products of partial ITS2A of 15 *An. minimus*. Lane M is the Gene Ruler 100 bp DNA ladder. B is a negative control. Lane 1-15 are PCR products of sample NO. 1-15.

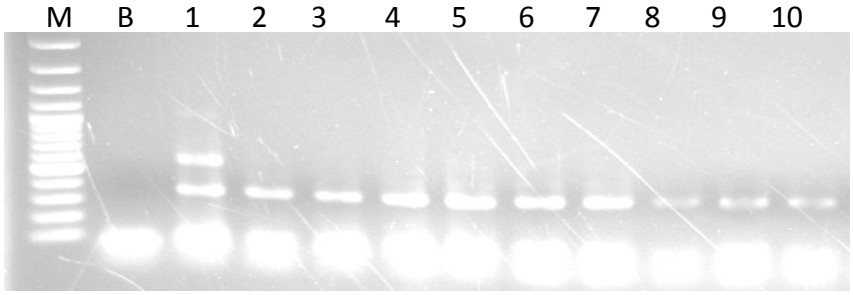

Figure 2 PCR products of partial ITS2A of 10 *An. dirus*. Lane M is a 100 bp DNA ladder. B is a negative control. Lane 1-10 are PCR products of sample NO. 1-10.

Detection of plasmodium infection

The DNA samples were used as templates to examine the infection of *Plasmodium*. Of 15 *An. minimus*, only two (sample No. 5 and 13) were infected with *Plasmodium* (Fig. 3), while one of 10 *An. scanloni* was infected with *Plasmodium* (Fig. 4).

Isolation and identification of bacteria in the midgut of *An. minimus* and *An. scanloni*

The midguts of *An. minimus* and *An. scanloni* were grinded in the sterilized saline solution and spread on LA plates. After 16 h, bacterial colonies derived from the midgut of six 15 *An. minimus* and four *An. scanloni* were observed. Single colony of different types of forms in each plate was picked up and spread on new LA plates. Colony or DNA of these bacteria was used as templates in PCR reaction. Amplification of partial 16S rRNA gene was successful except three bacteria samples, 9-1, 9-2 and 9-3 isolated from *An. minimus*. Identification of bacterial species isolated from the midgut of *An. minimus* and from the midgut of *An. scanloni* using blast program are presented in Table 1.

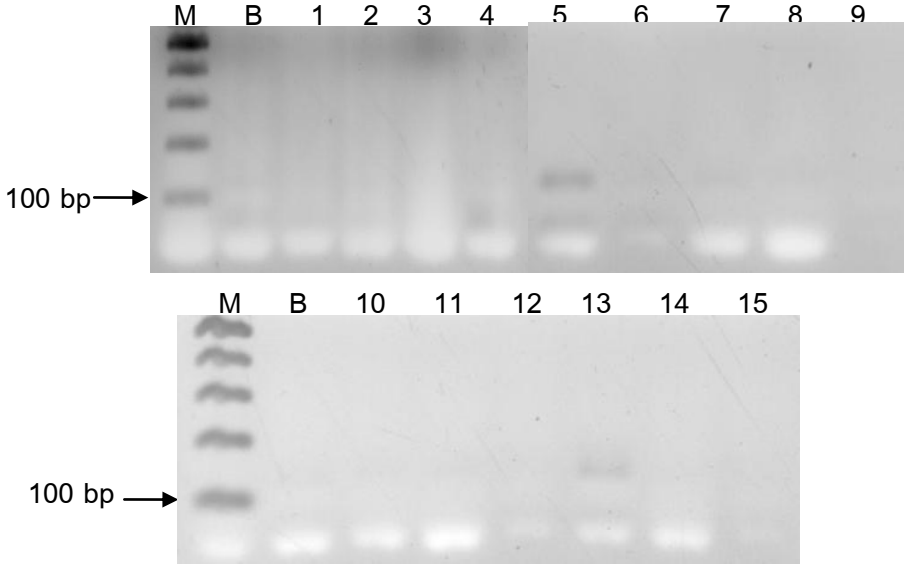

Figure 3 Detection of *Plasmodium* infection in *An. minimus*. Lane M is the Gene Ruler 100 bp DNA ladder. B is a negative control. Lane 1–15 are PCR products of sample NO. 1–15.

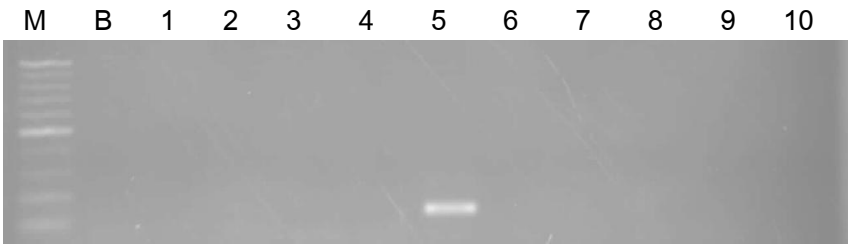

Figure 4 Detection of *Plasmodium* in *An. scanloni*. Lane M is the Gene Ruler 100 bp DNA ladder. B is a negative control. Lane 1–10 are PCR products of sample NO. 1–10.

Table 1 Identification of bacterial species isolated from the midgut of *An. minimus* and *An. scanloni* using blast program. Only the most similar bacterial species from GenBank database to our bacterial samples was presented.

Mosquito species	Code	Bacterial species
An. minimus	B6-1	*Candidatus Chryseobacterium*
	B7-2	*Cedecea davisae*
	B7-3	Uncultured bacterium clone
	B7-5	Uncultured *Acinetobacter sp.*
	B7-6	*Enterobacter sp.*
	B8-1	Uncultured *Paenibacillus sp.* clone
	B8-4	*Cellulosimicrobium cellulans*
	B9-2	*Paenibacillus uliginis*
	B9-3	*Paenibacillus uliginis*
	B13-1	*Acinetobacter sp.*
	B13-5	Uncultured bacterium
An. scanloni	M4-1	Unculture bacterium
	M7-1	*Staphylococcus epidermidis*
	M7-2	*Staphylococcus sp.*
	M7-3	*Staphylococcus epidermidis*
	M8-1	*Bacillus pumilus*
	M9-1	*Bacillus sp.*

Primer selection

We selected primers in the 16S rRNA gene from previous reports. Since V4 in the 16S rRNA gene has been reported as a better region for identifying bacterial species than the others (Boissere, et al., 2012), we selected two primer pairs (Table 2), 347F/803R and 338F/786R, located in the region. Then, these primers were added in the PCR reaction and DNA extracted

from one mosquito was used as a template. The result showed that only primer 347F/ 803R produced one DNA band with 500 bp long, but the primer 338F/786R produced at least two DNA bands (Fig. 5)

To make sure that the PCR products produced from the primer 347F/803R were partial fragments of bacterial 16S rRNA gene, they were cloned and sequenced. The blast result showed that nucleotide sequences of these PCR products were similar to those of 16S rRNA genes of bacteria and the max identification were ranged between 91%-99% (Table 3)

Table 2 Profile of primers used for identification of bacterial species in Metagenomics.

Primer name	Nucleotide sequence (5'->3')	Size of PCR product (base pair)
338F	ACT CCT ACG GGA GGC AGC AG	449
786R	GAC TAC CAG GGT ATC TAA TC	
347F	GGA GGC AGC AGT RRG GAA T	457
803R	CTA CCR GGG TAT CTA ATC C	

As a complementary approach, we also applied a culture-independent strategy to explore the microbial diversity in *An. minimus* midgut using 454 pyrosequencing. We obtained a total of 42,599 raw reads and after the adapter and low-quality trimming, 33,564 filtered reads (78.79%) were assigned the phylotypes using the RDP classifier. We were able to designate 99.95% of the reads as originating from bacteria, and 90.78% and 73.41% of the trimmed reads were assigned to bacteria at the family and the genus levels, respectively.

We also applied statistical models to assess the genotype richness and evenness of the *An. minimus* midgut metagenomes. Shannon index revealed that the Plasmodium-uninfected host contained greater numbers of bacterial species than the Plasmodium-infected host suggesting that the midgut microbiome of the uninfected mosquitoes display a higher degree of diversity compared to the Plasmodium-infected individuals (Table 4).

Taxonomic classification with RDP classifier detected the presence of 61 species in four bacterial phyla in the midgut of *An. minimus*. Proteobacteria was far more abundant than the other groups, representing 94.48% of the OTUs assigned and containing the 49 distinct genera (Table 5). At the class level, the midgut community was dominated by two taxonomic classes: Gammaproteobacteria (51.92%) and Betaproteobacteria (35.76%). Among members of the Gammaproteobacteria, *Moraxellaceae* was associated with 28.79% of the sequences while other predominant OTUs were assigned to *Enterobacteriaceae* (16.49%). For betaproteo-bacteria, the major bacterial groups identified were *Alcaligenaceae* (16.26%) and

Burkholderiaceae (15.79%). The most abundant genus (> 1,000 OTUs) was *Acinetobacter* (26.90%) followed by *Burkhoderia* (13.28%), *Alcaligenes* (12.89%) and *Serratia* (3.49%).

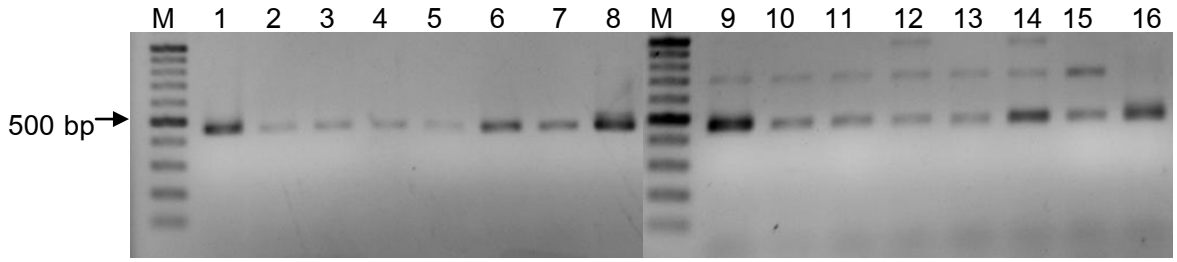

Figure 5 PCR products of partial 16S rRNA gene using two primer pairs. M is a 100 bp DNA ladder. Lanes 1-8 are PCR products produced from primer 347F/803R and Lanes 9-16 are PCR products produced from primer 33 F/786R.

Table 3 Identification of bacterial species using Blast program.

Bacterial species	Accession No.	Max identification
Pseudomonas mosselii	NR_024924	99%
Delftia acidovorans	NR_024711	91%
Pseudomonas mosselii	NR_024924	96%
Pseudomonas mosselii	NR_024924	99%
Enterobacter hormaechei	NR_042154	99%

From the total of 61 genera, 20 of them were found in both the infected and the uninfected mosquitoes while 8 and 33 genera were detected only in the *Plasmodium*-infected and the *Plasmodium*-uninfected ones, respectively. The majority of bacteria presented in the midgut of the infected host were gammaproteobacteria (96.17%), followed by betaproteo-bacteria (2.82%; Fig.6). *Moraxellaceae* (65.88%) and *Enterobacteriaceae* (27.45%) families were the most abundance in the gammaproteobacteria (Fig.6). *Acinetobacter* species (64.93%) represented the majority of the *Moraxellaceae* while *Thorsellia* species (4.24%) was the most prevalent in the *Enterobacteriaceae*.

In contrast, the midgut of uninfected mosquitoes was dominated with betaproteobacteria (58.67%; Fig.6). Moreover, Actinobacteria, alphaproteobacteria, Firmicutes and Bacteroidetes were almost exclusively found in the midgut of the uninfected mosquitoes. Among them, *Burkholderia* species (21.90%) in the family *Burkholderiaceae* and *Alcaligenes* species (21.88%) in the family *Alcaligeneceae* families were the most prevalent (Fig.6).

Table 4 Biodiversity indices of *Plasmodium*-infected and *Plasmodium*-uninfected *An. minimus*.

Sample	Richness			Shannon-Weaver index		
	0.03	0.05	0.15	0.03	0.05	0.15
Plasmodium-uninfected sample	3,182	1,387	281	7.23	6.03	3.94
Plasmodium-infected sample	1,373	560	101	5.98	4.65	2.60

Table 5 The 61 genera in the midgut of *Anopheles minimus*. Certain genera were detected in both *Plasmodium*-uninfected and infected mosquitoes (B) whereas some 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*	PU	0.06
	*Brachybacterium**	B	0.50
	Kocuria	PU	0.06
	Micrococcus	PU	1.14
	Propionibacterium	PU	0.15
Bacteroidetes	*Elizabethkingia*	PU	0.93
	Flavobacterium	B	0.01
	*Flavisolibacter**	PU	0.13
	*Pedobacter**	PU	<0.01
Firmicutes	*Bacillus*	PU	0.03
	Staphylococcus	B	0.67
	Clostridium sensu stricto	PU	0.01
Proteobacteria/ Alphaproteobacteria	*Brevundimonas*	PU	0.02
	Phenylobacterium	PU	<0.01
	*Bartonella**	PU	0.01
	Bradyrhizobium	PU	0.01
	*Nitrobacter**	PU	0.03
	Methylobacterium	B	0.07
	*Aquamicrobium**	PU	0.01
	*Mesorhizobium**	PU	0.04
	Rhizobium	PU	0.08
	Asaia	PU	0.01
	Roseomonas	PU	0.13
	Novosphingobium	B	0.11
	Sphingomonas	B	0.50
Proteobacteria/ Betaproteobacteria	*Achromobacter*	B	2.34
	Alcaligenes	PU	12.89

Phylum/class	Genus	Detection (B, PU, PI)	Percentage from total OTUs
	Bordetella	PU	0.04
	Burkholderia	B	13.28
	Ralstonia	PU	0.06
	Aquabacterium	PU	0.03
	Acidovorax	PU	0.06
	Comamonas	B	0.01
	Delftia	B	0.46
	*Tepidicella**	PI	<0.01
	*Variovorax**	PU	<0.01
	*Massilia**	B	0.24
	*Naxibacter**	PU	<0.01
	*Methyloversatilis**	PU	0.01
Proteobacteria/ Deltaproteobacteria	*Desulfohalobium**	PU	0.17
Proteobacteria/ Gammaproteobacteria	*Aeromonas*	PI	0.01
	*Pseudoalteromonas**	PU	0.32
	Citrobacter	PI	<0.01
	Enterobacter	PI	<0.01
	Escherichia/Shigella	PI	0.01
	Klebsiella	B	0.33
	Pantoea	PI	<0.01
	Salmonella	B	0.13
	Serratia	B	3.49
	*Shimwellia**	PI	0.01
	Thorsellia	B	1.75
	*Trabulsiella**	PI	<0.01
	Zymobacter	PU	0.02
	Acinetobacter	B	26.90
	*Alkanindiges**	PU	<0.01
	Enhydrobacter	B	1.48
	Pseudomonas	B	1.03
	*Lucibacterium**	PU	0.14
	Vibrio	PU	0.02
	Stenotrophomonas	B	2.23
	*Xanthomonas**	B	1.14

Note: The asterisk (*) was marked for the new genera detected in the midgut of *Anopheles* species for the first time.

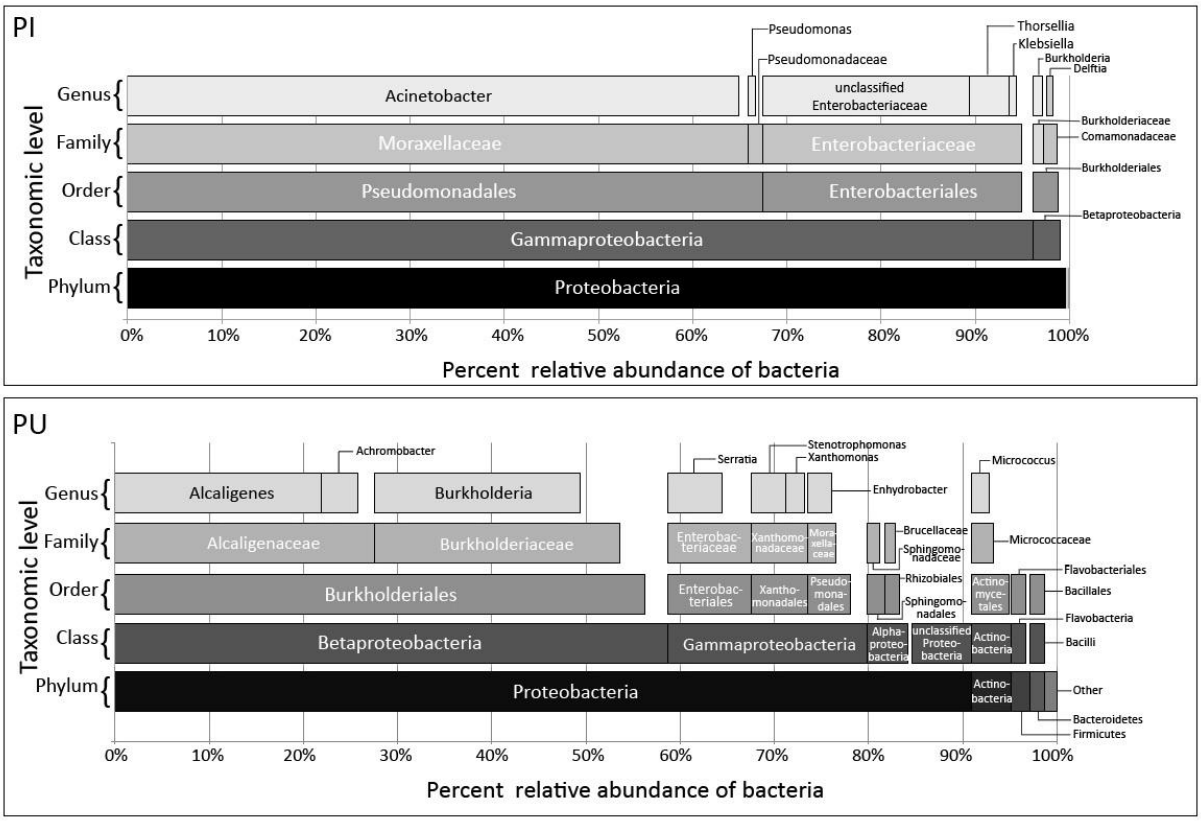

Figure 6 Percentages of relative abundance at taxa levels. (PI) and (PU) indicate the relative abundance (%) of bacteria in the midgut of the *Plasmodium*-infected and the uninfected *An. minimus*, respectively

6. Conclusion and Discussion

The mosquitoes we collected from Tak and Satul provinces are *An. minimus* and *An. scanloni,* respectively. Certain mosquitoes were infected with plasmodium. Bacterial colonies isolated from the midgut of six *An. minimus* A and four *An. scanloni* could be observed. Species of these bacteria were identified by PCR technique and analyzed by Blast program. Most of the colonies could be identified at genus or species levels, while three colonies are uncultured bacteria. Interestingly, *Enterobacter* sp. *and Acinetobacter* sp. isolated from the midgut of *An. minimus* have been reported that they were also isolated from the midgut of other *Anopheles* species. Moreover, *Enterobacter* sp. has been reported that it could inhibit the growth of Plasmodium.

Since isolation and identification of bacteria via the cultured method has limitation. Only small number of bacteria could grow on the synthetic media, the uncultured method can accomplish to overcome the cultured problem. Here, DNA extracted from the midgut of the mosquitoes was used as a template in PCR reaction. The PCR product was directly applied to

identify the bacterial species. In our study, we have got a primer pair, 347F/803R which was very effective to produce a partial fragment of 16S rRNA gene of bacteria. Then, we selected tags (eight oligonucleotides) to incorporate into the primer 347F/803R. These primers with a tag were used to prepare samples for identifying microbiota in the uncultured method and 61 bacterial genera were identified. Of 61, 19 genera have never been found in *Anopheles* species but were isolated from other insect guts, plant tissues, soils, air and water resources (França *et al*. 2006; Kalyuzhnaya *et al*. 2006; Chou *et al*. 2007; Shimelash *et al*. 2008; Huang *et al*. 2012; Bodenhausen *et al*. 2013; Maynaud *et al*. 2013; Yin *et al*. 2013). 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.

Mosquitoes contained numerous microorganisms especially in the gut (Azambuja *et al*. 2005). Several advantages of gut bacteria have been reported, including parasite-development inhibition (Bando *et al*. 2013). Touré *et al.* (2000) reported that antibiotic-treated *An. gambiae* and *An. stephensi* had lower bacteria contents and higher *P. falciparum* infection rates than the untreated mosquitoes. In addition, intrathoracic inoculation of bacteria in *An. gambiae* induced antibacterial peptide production that inhibited Plasmodium development (Lowenberger *et al*. 1999). Moreover, Cirimotich *et al*. (2011) 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* (Bando *et al*. 2013). These findings suggested that certain gram-negative bacteria play an important role to protect mosquitoes from Plasmodium infection.

In our stidy, the gram-negative bacteria, Acinetobacter, was the most abundant bacteria in the midgut samples. 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 (Bahia *et al*. 2014). In addition, the infection of Acinetobacter in the mosquito midgut reduced longevity of *An. gambiae* (Bahia *et al*. 2014). *Elizabethkingia meningoseptica* and *Serratia marcescens* were also detected in our work and they previously exhibited anti-Plasmodium activity in *Anopheles* species (Gonzalez-Ceron *et al*. 2003; Bando *et al*. 2013; Ngwa *et al*. 2013; Bahia *et al*. 2014). *E. meningoseptica* was able to inhibit the development of *P. falciparum* at the gametocyte transmission stage (Ngwa *et al*. 2013). Recently, Bahia et al. (2014) 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.

In addition, we could also genetic engineer symbiotic bacteria to make them have the ability to kill plasmodium. Here, there were only three genera, Burkholderia, Acinetobacter and Alcaligenes that contained relative abundant more than 10% from the total OTUs. The result showed that they grew and adapted well in the midgut of *An. minimus*. In the future, we might test if Burkholderia and Alcaligenes have ability to inhibit the growth of Plasmodium. If so, these bacteria could be use to malaria control directly. If not, they might be genetic engineered before use as a tool for malaria control.

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7. Appendix –

8. Output

This study was financially supported by the Kasetsart University Research and Development Institute and Thailand Research Fund National.

8.1 International Journal Publication

Surat W., W. Mhuantong, D. Sangsrakru, T. Chareonviriyaphap, U. Arunyawat, A. Kubera, T. Sittivicharpinyo, O. Siripan, W. Pootakham. Gut bacterial diversity in *Plasmodium*-infected and *Plasmodium*-uninfected *Anopheles minimus*. Chiang Mai Journal of Science *In Press*.

8.2 Application –

8.3 Others –

Chiang Mai J. Sci. 2015; 42(X) : 1-22
http://epg.science.cmu.ac.th/ejournal/
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) na□ ve 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

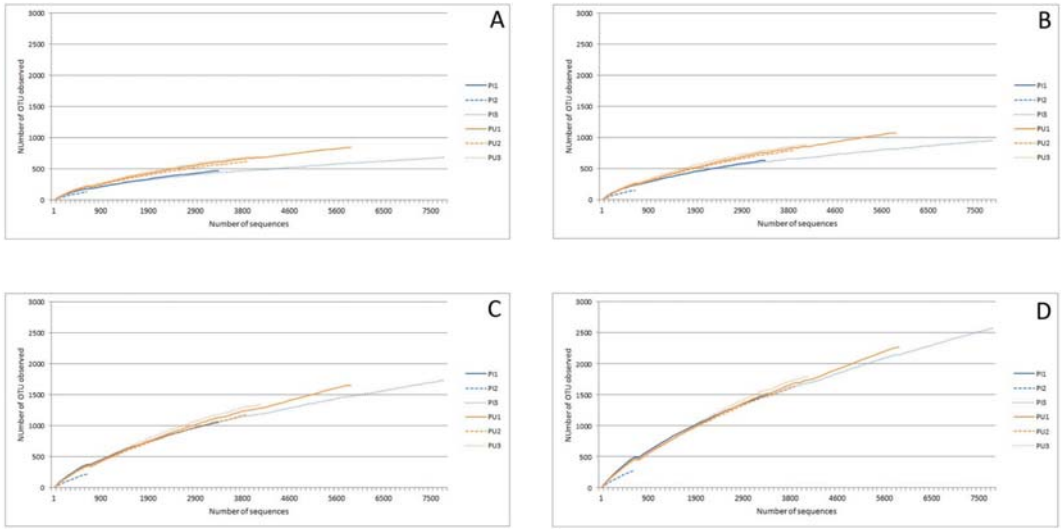

Figure 1. Rarefraction curves of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus* at four similarity levels; 85% (A), 90% (B), 95% (C) and 97% (D), respectively.

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).

Table S1 The number and the percentage of bacterial abundance in the gut of the *Plasmodium*-infected (PI) and those of the *Plasmodium*-uninfected (PU) *Anopheles minimus* at phylum, class, family and genus levels.

Taxonomy: Phylum	PI1	PI2	PI3	PU1	PU2	PU3	Total
Actinobacteria	0.00	0.00	0.03	8.78	0.41	0.30	2.11
Bacteroidetes	0.03	0.14	0.00	1.70	3.86	1.88	1.28
Cyanobacteria/Chloroplast	0.00	1.44	0.03	0.00	0.00	0.00	0.05
Firmicutes	0.00	0.14	0.05	2.11	1.06	1.51	0.90
Proteobacteria	99.97	98.13	99.75	85.55	94.38	95.51	95.02
Unclassified bacteria	0.00	0.14	0.11	1.78	0.28	0.72	0.60
Unclassified	0.00	0.00	0.04	0.09	0.00	0.07	0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Taxonomy: Class	PI1	PI2	PI3	PU1	PU2	PU3	Total
Actinobacteria;Actinobacteria	0.00	0.00	0.03	8.79	0.41	0.30	2.11
Bacteroidetes;Flavobacteria	0.03	0.14	0.00	0.75	3.86	1.88	1.06
Bacteroidetes;Sphingobacteria	0.00	0.00	0.00	0.94	0.00	0.00	0.21
Cyanobacteria/Chloroplast;Chloroplast	0.00	1.44	0.03	0.00	0.00	0.00	0.05
Firmicutes;Bacilli	0.00	0.14	0.05	2.11	1.06	1.51	0.90
Proteobacteria;Alphaproteobacteria	0.15	0.72	0.04	4.06	5.39	2.31	2.15
Proteobacteria;Betaproteobacteria	2.96	13.84	0.83	65.68	66.07	58.29	35.12
Proteobacteria;Deltaproteobacteria	0.00	0.00	0.00	1.11	0.36	0.00	0.31
Proteobacteria;Gammaproteobacteria	96.80	83.58	98.87	14.59	22.52	34.90	57.40
Proteobacteria;unclassified Proteobacteria	0.06	0.00	0.00	0.10	0.05	0.02	0.04
Unclassified bacteria	0.00	0.14	0.11	1.78	0.28	0.72	0.60
Unclassified	0.00	0.00	0.04	0.09	0.00	0.07	0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Taxonomy: Family	PI1	PI2	PI3	PU1	PU2	PU3	Total
Actinobacteria; Actinobacteria; Actinomycetales; *Corynebacteriaceae*	0.00	0.00	0.00	0.00	0.33	0.10	0.07

Table S1 Continued.

Taxonomy: Family	PI1	PI2	PI3	PU1	PU2	PU3	Total
Actinobacteria; Actinobacteria; Actinomycetales; *Dermabacteraceae*	0.00	0.00	0.03	2.02	0.00	0.00	0.50
Actinobacteria; Actinobacteria; Actinomycetales; *Micrococcaceae*	0.00	0.00	0.00	5.73	0.00	0.20	1.39
Actinobacteria; Actinobacteria; Actinomycetales; *Propionibacteriaceae*	0.00	0.00	0.00	0.75	0.00	0.00	0.17
Actinobacteria; Actinobacteria; Actinomycetales; unclassified Actinomycetales	0.00	0.00	0.00	0.27	0.05	0.00	0.35
Actinobacteria; Actinobacteria; unclassified Actinobacteria	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Bacteroidetes; Flavobacteria; Flavobacteriales; *Flavobacteriaceae*	0.03	0.14	0.00	0.75	3.86	1.88	0.97
Bacteroidetes; Sphingobacteria; Sphingobacteriales; *Chitinophagaceae*	0.00	0.00	0.00	0.94	0.00	0.00	0.22
Cyanobacteria/Chloroplast; Chloroplast; Chloroplast; Streptophyta	0.00	1.44	0.03	0.00	0.00	0.00	0.04
Firmicutes; Bacilli; Bacillales; *Bacillaceae* 1	0.00	0.00	0.03	0.00	0.28	0.30	0.10
Firmicutes; Bacilli; Bacillales; *Staphylococcaceae*	0.00	0.14	0.01	2.07	0.67	0.84	0.69
Firmicutes;Bacilli; Bacillales; unclassified Bacillales	0.00	0.00	0.01	0.02	0.10	0.37	0.12
Firmicutes;Bacilli;unclassified Bacilli	0.00	0.00	0.00	0.02	0.00	0.00	0.01
Proteobacteria;Alphaproteobacteria; Caulobacterales;*Caulobacteraceae*	0.00	0.00	0.00	0.00	0.15	0.00	0.04
Proteobacteria;Alphaproteobacteria; Rhizobiales;*Bradyrhizobiaceae*	0.00	0.00	0.00	0.02	0.75	0.05	0.10
Proteobacteria;Alphaproteobacteria; Rhizobiales;*Brucellaceae*	0.00	0.00	0.00	0.00	2.14	0.55	0.33
Proteobacteria;Alphaproteobacteria; Rhizobiales;*Methylobacteriaceae*	0.00	0.00	0.03	0.31	0.00	0.00	0.07
Proteobacteria;Alphaproteobacteria; Rhizobiales;*Phyllobacteriaceae*	0.00	0.00	0.00	0.00	0.59	0.00	0.10
Taxonomy: Family	PI1	PI2	PI3	PU1	PU2	PU3	Total
Proteobacteria;Alphaproteobacteria; Rhizobiales;*Rhizobiaceae*	0.00	0.00	0.00	0.38	0.10	0.00	0.08
Proteobacteria;Alphaproteobacteria; Rhizobiales;unclassified Rhizobiales	0.00	0.00	0.00	0.15	0.10	0.12	0.44
Proteobacteria;Alphaproteobacteria; Rhodobacterales;*Rhodobacteraceae*	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Proteobacteria;Alphaproteobacteria; Rhodospirillales; *Acetobacteraceae*	0.00	0.00	0.00	0.58	0.00	0.12	0.18

Table S1 Continued.

Taxonomy: Family	PI1	PI2	PI3	PU1	PU2	PU3	Total
Proteobacteria;Alphaproteobacteria; Sphingomonadales;*Sphingomonadaceae*	0.15	0.58	0.01	1.59	1.49	1.39	0.71
Proteobacteria;Alphaproteobacteria; Sphingomonadales;unclassified Sphingomonadales	0.00	0.00	0.00	0.82	0.03	0.00	0.26
Proteobacteria;Alphaproteobacteria; unclassified Alphaproteobacteria	0.00	0.14	0.00	0.21	0.03	0.05	0.31
Proteobacteria;Betaproteobacteria; Burkholderiales;*Alcaligenaceae*	0.00	0.72	0.04	11.42	51.20	47.15	16.26
Proteobacteria;Betaproteobacteria; Burkholderiales;*Burkholderiaceae*	0.06	12.68	0.23	49.48	13.91	9.85	15.79
Proteobacteria;Betaproteobacteria; Burkholderiales;*Burkholderiales incertae sedis*	0.00	0.00	0.00	0.00	0.26	0.00	0.03
Proteobacteria;Betaproteobacteria; Burkholderiales;*Comamonadaceae*	2.84	0.00	0.47	0.82	0.36	1.22	0.95
Proteobacteria;Betaproteobacteria; Burkholderiales;*Oxalobacteraceae*	0.00	0.00	0.06	2.73	0.00	0.02	0.55
Proteobacteria;Betaproteobacteria; Burkholderiales;unclassified Burkholderiales	0.03	0.00	0.01	0.24	0.03	0.00	0.68
Proteobacteria;Betaproteobacteria; Methylophilales;*Methylophilaceae*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Proteobacteria;Betaproteobacteria; Rhodocyclales;*Rhodocyclaceae*	0.00	0.00	0.00	0.00	0.05	0.00	0.01
Proteobacteria;Betaproteobacteria; unclassified Betaproteobacteria	0.03	0.43	0.00	0.99	0.26	0.05	1.48
Proteobacteria;Deltaproteobacteria; Desulfovibrionales;*Desulfohalobiaceae*	0.00	0.00	0.00	1.10	0.33	0.00	0.24
Proteobacteria;Deltaproteobacteria; Desulfovibrionales;unclassified Desulfovibrionales	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Proteobacteria;Deltaproteobacteria; unclassified Deltaproteobacteria	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Proteobacteria;Gammaproteobacteria; Aeromonadales;*Aeromonadaceae*	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Proteobacteria;Gammaproteobacteria; Alteromonadales;*Pseudoalteromonadaceae*	0.00	0.00	0.00	1.65	0.00	0.00	0.32
Proteobacteria;Gammaproteobacteria; Alteromonadales;unclassified Alteromonadales	0.00	0.00	0.00	0.15	0.00	0.00	0.05
Proteobacteria;Gammaproteobacteria; Enterobacteriales;*Enterobacteriaceae*	47.25	80.12	12.57	0.17	13.45	19.69	16.50

Table S1 Continued.

Taxonomy: Family	PI1	PI2	PI3	PU1	PU2	PU3	Total
Proteobacteria;Gammaproteobacteria; Oceanospirillales;*Halomonadaceae*	0.00	0.00	0.00	0.00	0.00	0.10	0.03
Proteobacteria;Gammaproteobacteria; Oceanospirillales;unclassified Oceanospirillales	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Proteobacteria;Gammaproteobacteria; Pseudomonadales;*Moraxellaceae*	48.24	3.03	84.45	5.61	0.98	1.41	28.79
Proteobacteria;Gammaproteobacteria; Pseudomonadales;*Pseudomonadaceae*	1.25	0.00	1.71	0.00	0.03	6.13	1.60
Proteobacteria;Gammaproteobacteria; Vibrionales;Vibrionaceae	0.00	0.00	0.00	1.01	0.00	0.00	0.28
Proteobacteria;Gammaproteobacteria; Xanthomonadales;*Xanthomonadaceae*	0.06	0.43	0.14	5.97	8.04	7.51	3.60
Proteobacteria;Gammaproteobacteria; Unclassified Gammaproteobacteria;	0.00	0.00	0.00	0.03	0.03	0.02	0.74
Proteobacteria;unclassified Proteobacteria	0.06	0.00	0.00	0.10	0.05	0.02	3.92
Unclassified bacteria	0.00	0.14	0.11	1.78	0.28	0.72	0.78
Unclassified	0.00	0.00	0.04	0.09	0.00	0.07	0.05
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Taxonomy: Genus	PI1						
Actinobacteria		PI2	PI3	PU1	PU2	PU3	Total
Actinomycetales;*Corynebacteriaceae;* Corynebacterium	0.00	0.00	0.00	0.00	0.33	0.10	0.07
Actinomycetales;*Dermabacteraceae;* Brachybacterium	0.00	0.00	0.03	2.02	0.00	0.00	0.47
Actinomycetales;*Micrococcaceae; Kocuria*	0.00	0.00	0.00	0.17	0.00	0.20	0.07
Actinomycetales;*Micrococcaceae; Micrococcus*	0.00	0.00	0.00	5.35	0.00	0.00	1.22
Actinomycetales;*Micrococcaceae*; unclassified Micrococcaceae	0.00	0.00	0.00	0.21	0.00	0.00	0.05
Actinomycetales;*Propionibacteriaceae;* Propionibacterium	0.00	0.00	0.00	0.69	0.00	0.00	0.16
Actinomycetales;*Propionibacteriaceae*; unclassified Propionibacteriaceae	0.00	0.00	0.00	0.07	0.00	0.00	0.02
Actinomycetales;unclassified Actinomycetales	0.00	0.00	0.00	0.27	0.05	0.00	0.07
Unclassified Actinobacteria	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Bacteroidetes							
Flavobacteria;Flavobacteriales;*Flavobacteriaceae;* Elizabethkingia	0.00	0.00	0.00	0.75	3.81	1.88	1.05
Flavobacteria;Flavobacteriales;*Flavobacteriaceae;* Flavobacterium	0.03	0.14	0.00	0.00	0.05	0.00	0.02
Sphingobacteria;Sphingobacteriales; *Chitinophagaceae*;Flavisolibacter	0.00	0.00	0.00	0.55	0.00	0.00	0.12

Table S1 Continued.

Sphingobacteria;Sphingobacteriales; *Chitinophagaceae*;unclassified *Chitinophagaceae*	0.00	0.00	0.00	0.39	0.00	0.00	0.09
Cyanobacteria/Chloroplast							
Chloroplast;Chloroplast;Streptophyta; unclassified Streptophyta	0.00	1.44	0.03	0.00	0.00	0.00	0.05
Firmicutes							
Bacilli;Bacillales;*Bacillaceae* 1;*Bacillus*	0.00	0.00	0.00	0.00	0.15	0.05	0.03
Bacilli;Bacillales;*Bacillaceae* 1;unclassified *Bacillaceae*	0.00	0.00	0.03	0.00	0.13	0.25	0.07
Bacilli;Bacillales;*Staphylococcaceae;Staphylococcus*	0.00	0.14	0.01	2.06	0.67	0.84	0.71
Bacilli;Bacillales;*Staphylococcaceae*;unclassified *Staphylococcaceae*	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Bacilli;Bacillales;unclassified Bacillales	0.00	0.00	0.01	0.02	0.10	0.37	0.08
Bacilli;unclassified_Bacilli	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Proteobacteria; Alphaproteobacteria							
Caulobacterales;*Caulobacteraceae; Brevundimonas*	0.00	0.00	0.00	0.00	0.15	0.00	0.02
Rhizobiales;*Bradyrhizobiaceae; Bradyrhizobium*	0.00	0.00	0.00	0.02	0.00	0.05	0.01
Rhizobiales;*Bradyrhizobiaceae; Nitrobacter*	0.00	0.00	0.00	0.00	0.23	0.00	0.04
Rhizobiales;*Bradyrhizobiaceae*; unclassified *Bradyrhizobiaceae*	0.00	0.00	0.00	0.00	0.52	0.00	0.08
Rhizobiales;*Brucellaceae*; unclassified *Brucellaceae*	0.00	0.00	0.00	0.00	2.14	0.55	0.41
Rhizobiales;*Methylobacteriaceae; Methylobacterium*	0.00	0.00	0.03	0.31	0.00	0.00	0.08
Rhizobiales;*Phyllobacteriaceae; Mesorhizobium*	0.00	0.00	0.00	0.00	0.28	0.00	0.04
Rhizobiales;*Phyllobacteriaceae*; unclassified *Phyllobacteriaceae*	0.00	0.00	0.00	0.00	0.31	0.00	0.05
Rhizobiales;*Rhizobiaceae; Rhizobium*	0.00	0.00	0.00	0.38	0.10	0.00	0.10
Rhizobiales;unclassified Rhizobiales	0.00	0.00	0.00	0.15	0.10	0.12	0.07
Rhodobacterales;*Rhodobacteraceae*; unclassified *Rhodobacteraceae*	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Rhodospirillales;*Acetobacteraceae; Asaia*	0.00	0.00	0.00	0.00	0.00	0.05	0.01
Rhodospirillales;*Acetobacteraceae; Roseomonas*	0.00	0.00	0.00	0.57	0.00	0.00	0.13
Rhodospirillales;*Acetobacteraceae*; unclassified *Acetobacteraceae*	0.00	0.00	0.00	0.02	0.00	0.07	0.02
Sphingomonadales;*Sphingomonadaceae; Novosphingobium*	0.00	0.00	0.01	0.55	0.00	0.00	0.13
Sphingomonadales;*Sphingomonadaceae; Sphingomonas*	0.15	0.58	0.00	0.81	1.31	1.24	0.61

Table S1 Continued.

Sphingomonadales; *Sphingomonadaceae*; unclassified *Sphingomonadaceae*	0.00	0.00	0.00	0.24	0.18	0.15	0.11
Sphingomonadales; unclassified Sphingomonadales	0.00	0.00	0.00	0.82	0.03	0.00	0.19
unclassified Alphaproteobacteria	0.00	0.14	0.00	0.21	0.03	0.05	0.06
Taxonomy: Genus	PI1	PI2	PI3	PU1	PU2	PU3	Total
Proteobacteria;Betaproteobacteria							
Burkholderiales;*Alcaligenaceae; Achromobacter*	0.00	0.58	0.04	4.10	4.02	5.78	2.48
Burkholderiales;*Alcaligenaceae; Alcaligenes*	0.00	0.00	0.00	5.93	46.04	40.53	14.69
Burkholderiales;*Alcaligenaceae*; unclassified *Alcaligenaceae*	0.00	0.14	0.00	1.39	1.13	0.84	0.62
Burkholderiales;*Burkholderiaceae; Burkholderia*	0.06	12.39	0.20	47.06	13.09	9.50	14.59
Burkholderiales;*Burkholderiaceae; Ralstonia*	0.00	0.00	0.00	0.02	0.39	0.00	0.06
Burkholderiales;*Burkholderiaceae*; unclassified *Burkholderiaceae*	0.00	0.29	0.03	2.40	0.44	0.35	0.68
Burkholderiales;*Burkholderiales incertae sedis; Aquabacterium*	0.00	0.00	0.00	0.00	0.26	0.00	0.04
Burkholderiales;*Comamonadaceae; Acidovorax*	0.00	0.00	0.00	0.36	0.00	0.00	0.08
Burkholderiales;*Comamonadaceae; Comamonas*	0.06	0.00	0.00	0.00	0.00	0.02	0.01
Burkholderiales;*Comamonadaceae; Delftia*	2.12	0.00	0.18	0.00	0.36	0.79	0.51
Burkholderiales;*Comamonadaceae; Tepidicella*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Burkholderiales;*Comamonadaceae; Variovorax*	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Burkholderiales;*Comamonadaceae*; unclassified *Comamonadaceae*	0.66	0.00	0.28	0.45	0.00	0.40	0.34
Burkholderiales;*Oxalobacteraceae; Massilia*	0.00	0.00	0.05	1.30	0.00	0.00	0.31
Burkholderiales;*Oxalobacteraceae; Naxibacter*	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Burkholderiales;*Oxalobacteraceae*; unclassified *Oxalobacteraceae*	0.00	0.00	0.01	1.42	0.00	0.00	0.33
Burkholderiales;unclassified_Burkholderiales	0.03	0.00	0.01	0.24	0.03	0.00	0.07
Methylophilales;*Methylophilaceae*; unclassified *Methylophilaceae*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Rhodocyclales;*Rhodocyclaceae; Methyloversatilis*	0.00	0.00	0.00	0.00	0.05	0.00	0.01
unclassified Betaproteobacteria	0.03	0.43	0.00	0.99	0.26	0.05	0.29
Proteobacteria;Deltaproteobacteria							
Desulfovibrionales;*Desulfohalobiaceae; Desulfohalobium*	0.00	0.00	0.00	0.84	0.23	0.00	0.23
Desulfovibrionales;*Desulfohalobiaceae*; unclassified *Desulfohalobiaceae*	0.00	0.00	0.00	0.26	0.10	0.00	0.07
Desulfovibrionales; unclassified Desulfovibrionales;	0.00	0.00	0.00	0.00	0.03	0.00	0.00
unclassified Deltaproteobacteria	0.00	0.00	0.00	0.02	0.00	0.00	0.00

Table S1 Continued.

Taxonomy: Genus	PI1	PI2	PI3	PU1	PU2	PU3	Total
Proteobacteria;Gammaproteobacteria							
Aeromonadales;*Aeromonadaceae; Aeromonas*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Alteromonadales;*Pseudoalteromonadaceae; Pseudoalteromonas*	0.00	0.00	0.00	1.63	0.00	0.00	0.37
Alteromonadales; *Pseudoalteromonadaceae*; unclassified *Pseudoalteromonadaceae*	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Alteromonadales; unclassified Alteromonadales	0.00	0.00	0.00	0.15	0.00	0.00	0.04
Enterobacteriales;*Enterobacteriaceae; Citrobacter*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Enterobacteriales;*Enterobacteriaceae; Enterobacter*	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacteriales;*Enterobacteriaceae*; *Escherichia/Shigella*	0.00	0.58	0.00	0.00	0.00	0.00	0.02
Enterobacteriales;*Enterobacteriaceae; Klebsiella*	1.37	0.00	0.74	0.00	0.00	0.02	0.41
Enterobacteriales;*Enterobacteriaceae; Pantoea*	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Enterobacteriales;*Enterobacteriaceae; Salmonella*	1.04	0.00	0.03	0.00	0.08	0.00	0.16
Enterobacteriales;*Enterobacteriaceae; Serratia*	0.00	2.02	0.04	0.14	10.69	14.14	3.94
Enterobacteriales;*Enterobacteriaceae; Thorsellia*	0.00	76.66	0.00	0.02	0.00	0.02	2.08
Enterobacteriales;*Enterobacteriaceae; Trabulsiella*	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacteriales;*Enterobacteriaceae*; unclassified *Enterobacteriaceae*	44.78	0.86	11.74	0.02	2.68	5.51	10.74
Oceanospirillales;*Halomonadaceae; Zymobacter*	0.00	0.00	0.00	0.00	0.00	0.10	0.02
Oceanospirillales; unclassified Oceanospirillales	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Pseudomonadales;*Moraxellaceae; Acinetobacter*	47.82	2.88	84.12	0.00	0.82	1.07	32.37
Pseudomonadales;*Moraxellaceae; Enhydrobacter*	0.00	0.14	0.00	5.61	0.15	0.27	1.35
Pseudomonadales;*Moraxellaceae*; unclassified *Moraxellaceae*	0.42	0.00	0.33	0.00	0.00	0.07	0.17
Pseudomonadales;*Pseudomonadaceae; Pseudomonas*	1.10	0.00	0.84	0.00	0.03	5.03	1.20
Pseudomonadales;*Pseudomonadaceae*; unclassified *Pseudomonadaceae*	0.15	0.00	0.87	0.00	0.00	1.09	0.46

Table S1 Continued.

Vibrionales;*Vibrionaceae; Lucibacterium*	0.00	0.00	0.00	0.38	0.00	0.00	0.09
Vibrionales;*Vibrionaceae;Vibrio*	0.00	0.00	0.00	0.10	0.00	0.00	0.02
Vibrionales;*Vibrionaceae*; unclassified *Vibrionaceae*	0.00	0.00	0.00	0.53	0.00	0.00	0.12
Xanthomonadales;*Xanthomonadaceae; Stenotrophomonas*	0.06	0.00	0.14	2.11	6.65	5.01	2.32
Xanthomonadales;*Xanthomonadaceae*; *Xanthomonas*	0.00	0.43	0.00	3.65	1.08	2.28	1.37
Xanthomonadales;*Xanthomonadaceae*; unclassified Xanthomonadaceae	0.00	0.00	0.00	0.21	0.31	0.22	0.13
Unclassified Gammaproteobacteria	0.00	0.00	0.00	0.03	0.03	0.02	0.02
Unclassified Proteobacteria	0.06	0.00	0.00	0.10	0.05	0.02	0.04
Unclassified bacteria	0.00	0.14	0.11	1.78	0.28	0.72	0.60
Unclassified	0.00	0.00	0.04	0.09	0.00	0.07	0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

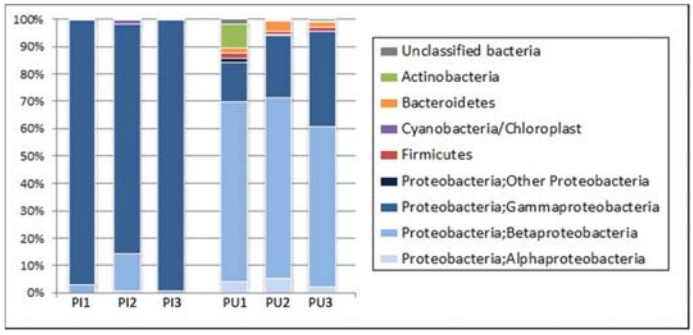

Figure 2. Taxonomic classification of microbiota in the gut of three *Plasmodium*-infected (PI; PI1, PI2, PI3) and three *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

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.

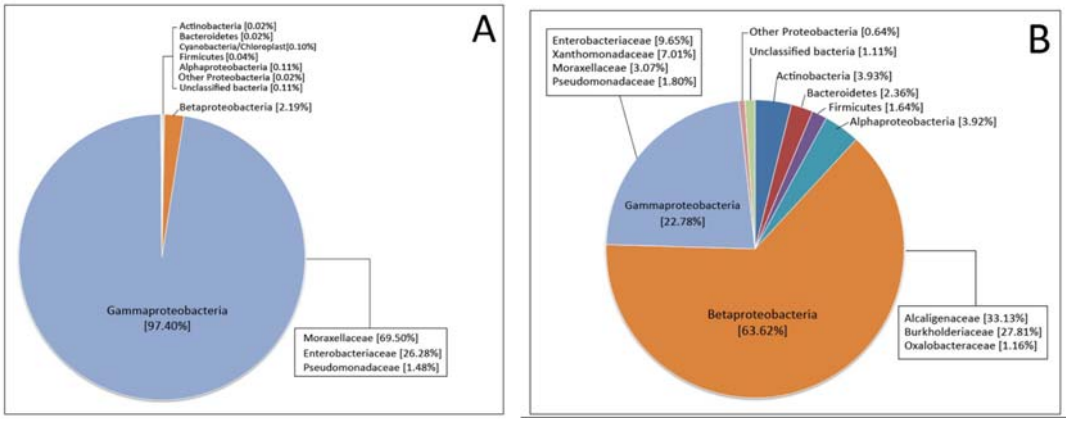

Figure 4. Percentages of relative abundance at taxonomic levels. (A) and (B) indicate the relative abundance (%) of bacteria in the gut of the *Plasmodium*-infected and the *Plasmodium*-uninfected *An. minimus*, respectively.

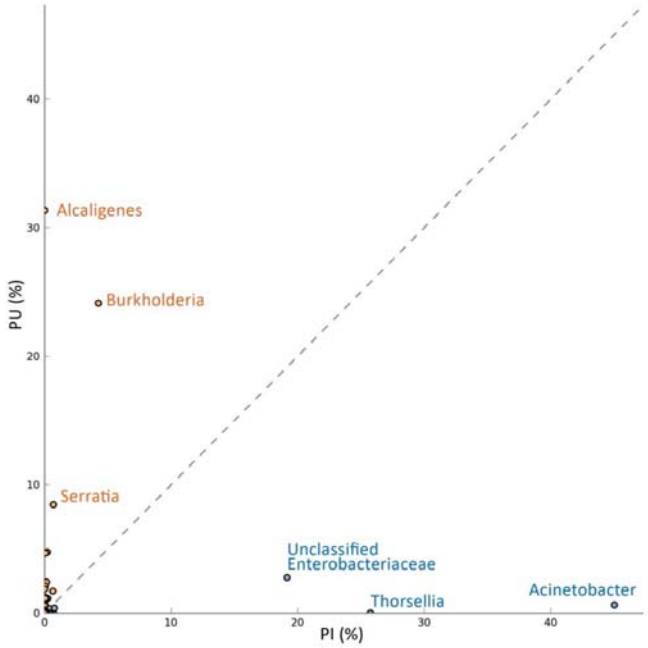

Figure 5. Scatter plot of prevalent bacterial genera comparing between the gut microbiota in the *Plasmodium*-infected (PI; PI1, PI2, PI3) and the *Plasmodium*-uninfected (PU; PU1, PU2, PU3) *An. minimus*.

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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