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Midgut bacterial diversity analysis of laboratory reared and wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in Kenya

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Midgut symbiotic bacteria are known to play fundamental roles in the biology of mosquitoes, however knowledge of midgut bacterial communities associated with mosquitoes is scanty due to limitation of the isolation techniques based on culturing. In this study, the composition and diversity of midgut bacteria in field collected and lab reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes was explored using the Illumina sequencing. Deoxyribonucleic acid was isolated from the pooled midgut extracts and their 16S rRNA gene sequenced using Illumina sequencing platform. Operational taxonomic units (OTUs) were analyzed using QIIME 1.8.0; taxonomy was assigned using BLASTn against SILVA 119 and hierarchical clustering was done using R program software. Out of the total number of sequence reads obtained, 145 OTUs were realized at 3% genetic distance. The 145 OTUs spanned 12 phyla; *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Euryarchaeota*, *Gemmatimonadetes*, *Spirochaetae*, *Archeabacteria* *Verrucomicrobia*, *Chloroflexi*, *Bacteriodetes*, *Acidobacteria* and *Actinobacteria*. Microbial community composition based on OTUs showed significant difference between field collected and lab reared mosquitoes (χ^2 = 45.0799, p = 3.2 × 10^{-5}). Similarly, there was a significant difference in community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2 = 31.2257, p = 7.7 × 10^{-4}). The bacterial composition and diversity appeared to be influenced by the environment and the species of the mosquitoes.

Key words: *Anopheles gambiae*, *Culex quinquefasciatus,* midgut, DNA, diversity.

INTRODUCTION

Mosquitoes transmit diseases like malaria, dengue, lymphatic filariasis, yellow fever among others. Among these diseases, malaria is the most important mosquito borne disease with an estimated 214 million new cases of malaria worldwide (World Health Organization (WHO), 2015). The African region accounted for most of the global cases of malaria (88%), followed by South-East Asia region (10%) and Eastern Mediterranean region (2%) (WHO, 2015). In Kenya, there were an estimated 6.7 million new clinical cases and 4,000 deaths each year

and those living in Western Kenya have an especially high risk of malaria (Centers for Disease Control and Prevention (CDC), 2015). Most of the deaths are caused by the parasite *Plasmodium falciparum* whose major vector in Africa is the mosquito species *Anopheles gambiae* that is widely distributed throughout the Afro-tropical belt (Boissière et al., 2012).

Another mosquito species *Culex quinquefasciatus* is the principal vector for *Wuchereria bancrofti*, the filarial worm that causes filariasis and Japanese encephalitis (Agrawal and Sashindran, 2006). Lymphatic filariasis is a major public health problem worldwide. It is estimated that 1.3 billion people from 83 countries are living with the disease or are at risk of infection (Agrawal and Sashindran, 2006). Lymphatic filariasis is present on the East African coast especially in Kenya (Njenga et al., 2011).

Current mosquito vector control strategies include insecticide treatment delivered through spraying houses and insecticide-impregnated mosquito nets. While these methods are effective at decreasing mosquito vector numbers, they have also contributed to the rise in insecticide resistant mosquitoes (Bando et al., 2013).

Various alternative approaches are being tried to reduce malaria cases in the world, and one such approach is paratransgenesis. Paratransgenesis is a method where by a symbiotic bacteria is used to express effector molecules inside a targeted vector. The symbiotic bacteria are genetically modified to produce effector molecules and then reintroduced into the mosquito to produce the required effect (Chavshin et al., 2012). Understanding the microbial community structure of the mosquito midgut is therefore necessary in order to identify possible bacterial candidates for paratransgenesis. The mosquito midgut plays a critical role to the survival and development of the parasites and is therefore, the most attractive site to target malaria parasites (Whitten et al., 2006). The midgut microbiota of mosquitoes is still not well investigated and a few studies have been carried out on microflora of wild caught malaria vectors (Wang et al., 2011). The available conventional culture techniques limit the scope in determination of the microbial diversity since it sometimes misses out on non-culturable microbes (Pidiyar et al., 2004).

In laboratory-raised mosquitoes, the midgut bacteria can be acquired through contaminated sugar solutions, blood meals and transmitted transstadially. However, in wild mosquitoes, the origin of the midgut bacteria, is still unknown (Riehle and Jacobs-Lorena, 2005). In the current study the bacterial composition and diversity in the midgut of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes were determined using the illumina sequencing.

MATERIALS AND METHODS

Study site

The study area for field collected mosquitoes was Ahero, Kenya, which is a malaria endemic region. It is located at latitude 0º 11'S and longitude 34º 55'E and is approximately 1153 m above sea level. The area has a tropical climate with significant rainfall throughout the year and with an average temperature of 23.0°C.

Collection of field *A. gambiae* and *C. quinquefasciatus* mosquitoes

Adult *A. gambiae* and *C. quinquefasciatus* mosquitoes were captured from pit shelters by use of Centre for Disease Control and Prevention (CDC) light traps. The CDC light traps were hung at least one meter above the ground on a tree or pole between 6:00 and 7:00 pm in the evening and left overnight. The collection bags containing the mosquitoes were picked between 6.00 and 6.30 am in the morning. The mosquitoes were then put into vial/jars from the collection bags using mouth aspirators and stored at 4°C. One hundred and thirty eight adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were identified to species level using a standard morphological key according to Gillies and De Meillon, (1968). The specimens were then transferred to the laboratory at the Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Acquisition of laboratory-reared *A. gambiae* and *C. quinquefasciatus*

One hundred and thirty eight laboratory reared adult female *A. gambiae* and *C. quinquefasciatus* mosquitoes were acquired from the International Centre of Insect Physiology and Ecology (ICIPE) Kasarani, Nairobi. They were transferred live to the laboratory at the Institute for Biotechnology Research, (JKUAT) and maintained in a mosquitarium at 28°C and 70 to 80% humidity until dissection. The mosquitoes were offered resins and 1% glucose solution as a source of energy and were not fed on blood.

Dissection of mosquitoes and isolation of DNA

Dissection of mosquitoes was done according to Rani et al. (2009). Before dissecting, the mosquitoes were chilled to death and sterilized with 70% ethanol then transferred into sterile distilled water in a sterile hood. The mosquitoes were dissected individually under sterile conditions. The midguts were mashed and suspended in 100 µl of sterile phosphate buffered solution (PBS). The mashed midguts were ground to homogeneity. Each midgut extract consisted of 20 pooled midguts of adult female mosquitoes. Field collected and lab reared mosquitoes had seven pooled midgut extracts each. The midgut extracts were stored at -80ºC until further analysis.

The total microbial genomic DNA was extracted separately from each group of mosquito midgut extracts using purelink genomic DNA mini kit (Invitrogen), following the manufacturer's

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instructions (CAT number, K1820-02 Life technologies, California, USA). Genomic DNA concentration was quantified using a nano drop spectrophotometer and the DNA stored at -20°C until further analysis.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene V4 variable region was carried out on the extracted DNA using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) that had a barcode (Caporaso et al., 2010). PCR amplification was carried out in 30 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min of intitial heating, followed by 30 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. PCR products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their DNA concentrations from the gel images. Pooled samples were purified using calibrated Ampure XP beads (Agencort Bioscience Corporation, MA, USA).

Amplicon library preparation

The pooled and purified PCR products were used to prepare DNA library by following Illumina TruSeq DNA library protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines. The resulting raw sequences were submitted to NCBI (Sequence Read Archive) with the following study accession numbers; sequences for field collected *A. gambiae* SAMN04386463; field collected *C. quinquefasciatus* SAMN04386464; lab reared *A. gambiae* SAMN04386465 and lab reared *C. quinquefasciatus* SAMN04386466.

Sequence analysis and taxonomic classification

Sequences obtained from the Illumina sequencing platform were depleted of barcodes and primers using a proprietary pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Short sequences < 200 bp, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6 bp were removed. The sequences were denoised, chimeras and singleton sequences removed (Capone et al., 2011; Dowd et al., 2011; Eren et al., 2011). De novo OTU clustering was done with standard UCLUST method using the default settings as implemented in QIIME Version 1.8.0 at 97% similarity level (Caporaso et al., 2010). Taxonomy was assigned to each OTU using BLASTn against SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013).

Diversity indices

Diversity indices (Shannon, Inverse Simpson, Evenness), rarefaction, Venn diagram (to compare the shared OTUs between the samples of mosquitoes) and hierarchical clustering were computed, using Vegan package version 1.16 to 32 in R software (R development Core Team, (2012). Kruskal-Wallis rank sum test was used to compare the relative abundance of gut microflora among *A. gambiae* and *C. quinquefasciatus* from lab reared and field collected samples using R programming language (R development Core Team (2012). Significance was tested at 95% confidence interval (p = 0.05). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to species using the taxa_summary.txt output from QIIME pipeline Version 1.8.0.

RESULTS

Assemblage and diversity of the microbial communities

After removing chimeras, denoising and demutiplexing, a total of 24,025 sequence reads greater than 200 bp were attained from the 16S rRNA data. Total OTU richness at 3% distance amounted to 145. The OTUs per data set ranged between 26 and 102. OTUs comprised 87% bacteria, 0.7% Archaea, 2% Fungi, 1.4% Eukarya and 8% no blast hit (sequences reads that were not assigned). Rarefaction curve was plotted in order to evaluate if all the diversity within the samples had been exhaustively recovered (Figure 1).

The slopes of the curves flatten out in cases where full diversity has been detected. This indicates that even if more sequences were obtained, the number of OTUs detected in the samples would not increase. However, more sequences would be required to exhaust the full diversity within the samples if the slopes did not flatten out (Chao et al., 2014). The sequencing depth as shown by the rarefaction curve was exhaustive enough to ensure the inclusion of the entire diversity of the microbes in the midgut of the two species of mosquitoes collected from field and lab reared.

The distribution of shared OTUs across the two species of mosquitoes and the sample source (lab reared and field collected) is shown in (Figure 2). Seven OTUs were common in all the samples, fifty four (54) OTUs were only found in field collected *A. gambiae* while 18 OTUs were detected only from the field collected *C. quinquefasciatus* samples. Lab reared *C. quinquefasciatus* and *A. gambiae* samples had one and 10 unshared OTUs, respectively.

A diversity index is a quantitative measure that reflects how many different types of species there are in a community and simultaneously takes into account how evenly the individuals are distributed among them.The estimated Shannon diversity index varied between (3.54) for field collected *A. gambiae* and (1.93) for lab reared *C. quinquefasciatus* (Table 1). The Shannon diversity index for field collected *C. quinquefasciatus* (2.73) was higher than lab reared *A. gambiae* (2.52) and lab reared *C. quinquefasciatus* (1.93). The Shannon index is a representation of species abundance and evenness, when either of these two factors increases, the diversity index also increases. Evenness index was used to estimate how well the species are evenly distributed in a community. The highest evenness was recorded from field collected *A. gambiae* (0.767) indicating that OTUs were evenly distributed as compared to other samples. The lowest evenness was recorded from lab reared *C.*

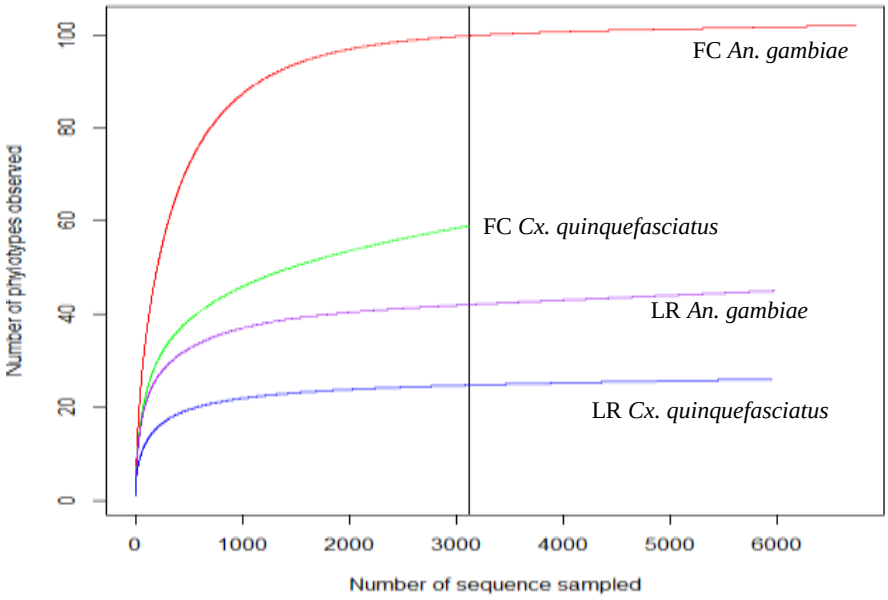

Figure 1. Rarefaction curve analysis in field collected (FC) *Cx.* (*Culex*) and lab reared (LR) *An.* (*Anopheles*) samples.

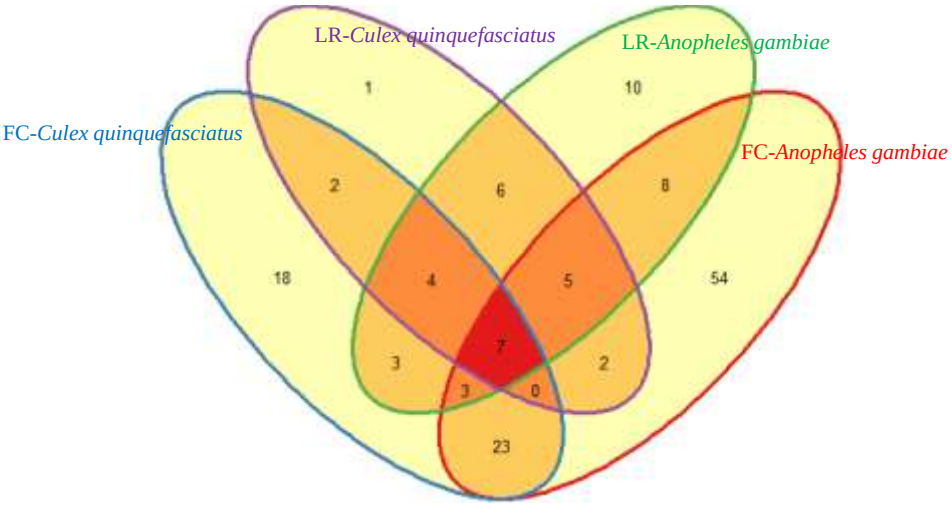

Figure 2. Venn diagram showing the distribution of shared OTUs across the 4 samples. Numbers indicate OTUs enumerated in field collected (FC) and lab reared (LR) samples.

Table 1. Diversity indices computed at OTU-based bacterial taxonomic units obtained from samples of the Field collected (FC) and Lab reared (LR) mosquitoes.

Sample	No. of sequences after filtering	Richness (OTUs)	Shannon (H)	Inverse Simpson	Evenness (J)	Effective no. of sp.
FC *Anopheles gambiae*	7516	102	3.54	19.98	0.767	34.47
FC *Culex quinquefasciatus*	3465	59	2.73	8.72	0.67	15.33
LR *Anopheles gambiae*	6669	45	2.52	5.98	0.661	12.43
LR *Culex quinquefasciatus*	6375	26	1.93	4.65	0.593	6.89
Total	**24,505**	**145**				

The microbial community composition, based on Kruskal-Wallis test, at OTU level showed significant difference between field collected and lab reared mosquitoes (χ^2= 45.0799, p = 3.2 × 10^{-5}). Similarly, there was significant difference in microbial community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2= 31.2257, p = 7.7 × 10^{-4}).

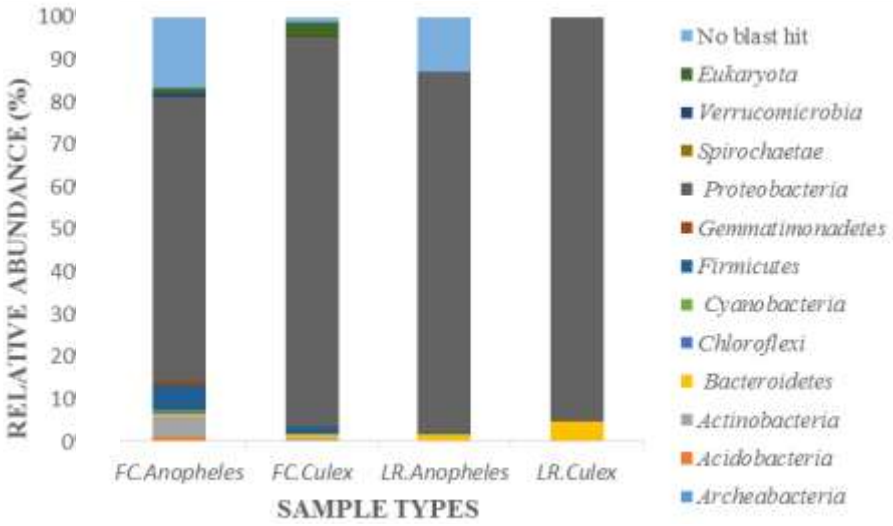

Figure 3. Relative abundance at phylum level from the field collected (FC) and lab reared (LR) samples.

quinquefasciatus (0.593) indicating that bacterial species are less evenly distributed and some species are more dominant than the others. The value of Inverse Simpson index ranged from 4.65 for lab reared *C. quinquefasciatus* to 19.98 for field collected *A. gambiae*. The value of Inverse Simpson index was observed to increase with increase in diversity**.**

Microbial taxonomic community composition

The SILVA SSU Reference 119 database (Quast et al., 2013) was used to assign reads to appropriate taxonomic ranks. The OTUs spanned 12 phyla (Figure 3); *Proteobacteria* (62.04-95.11 %)*, Firmicutes* (0.00-6.13 %), *Bacteriodetes* (0.42-4.89 %), *Actinobacteria* (0.00-4.97%), *Eukaryota* (0.00-3.46%), *Gemmatimonadetes* (0.00-0.86%), *Spirochaetae* (0.00-0.21%), *Verrucomicrobia* (0.00-0.76%), *Chloroflexi* (0.00-0.80%), *Acidobacteria* (0.00-0.68%), *Archeabacteria* (0.00-0.39%) and *Cyanobacteria* (0.00-0.10%). The no blast hits had relative abundance ranging from 0.00 to 16.58%.

OTUs belonging to the Phylum *Proteobacteria* were the most abundant and were represented by the most genera as shown in Figure 4. In lab reared *C. quinquefasciatus* sample the OTUs were affiliated to following genera; *Aeromonas*, *Asaia*, *Elizabethkingia*, *Enterobacter*, *Pseudomonas*, *Rahnella*, *Serratia* and *Wolbachia*. *Serratia marcescens* was the most abundant species in this sample with a relative abundance of 64.29%. Other species present in higher abundance were *Rahnella* uncultured bacterium 18.13%, *Serratia* uncultured bacterium 5.08%, *Wolbachia Embioptera* sp. 4.37% and *Elizabethkingia meningoseptica* 4.88% (Figure 4). However, in field collected *Culex quinquefasciatus* sample genera represented were, *Wolbachia*, *Sphingomonas*, *Streptococcus*, *Serratia*, *Rhizobium*, *Rahnella*, *Pseudomonas*, *Methylobacterium*, *Ixodes*, *Helicobacter*, *Gamma proteobacterium*, *Enterobacter*, *Corynebacterium*, *Bartonella*, *Bacteroidetes*, *Bacillus*, *Asaia*, *Arcobacter*, *Akkermansia*, *Agrobacterium*, and *Aeromonas*. The most abundant species in field collected *C. quinquefasciatus* sample were *Arcobacter* uncultured bacterium with relative abundance of 34.83%, while *Bartonella grahamii* as4aup had 24.45% (Figure 4). *Arcobacter* uncultured bacterium, *Bacteroidetes* uncultured bacterium, *B. grahamii* as4up, *Gamma Proteobacteria* uncultured bacterium, *Helicobacter* sp. B52Seymour and *Ixodes scapularis* were unique species in the field collected *C. quinquefasciatus* sample.

In lab reared *A. gambiae* sample, *Asaia* uncultured bacterium was the most abundant species with 39.30% relative abundance. Other taxa represented in the sample include *Aeromonas* sp. DMA1, *Rahnella* uncultured bacterium and *Serratia marcescens* each scoring a relative abundance of 10%. The genera found in lab reared *Anopheles gambiae* sample include; *Aeromonas, Serratia, Bacillus*, *Asaia, Chryseobacterium, Gluconacetobacter, Delftia, Pseudomonas, Rahnella, Thorsellia, Enterobacter* and *Stenotrophomonas*. *Thorsellia anophelis* was unique to lab reared *A. gambiae* sample (Figure 5). The field collected *A. gambiae* sample was found to harbor a higher diversity of bacterial species. The most abundant species were *Agrobacterium* sp. 12.63% and *Methylobacterium* uncultured bacterium at 11.14% relative abundance. The most predominant genera found in field collected include; *Serratia, Bacillus*, *Agrobacterium Stenotrophomonas, Gluconacetobacter, Methylobacterium*. *Rahnella* (Figure 5). The unique species in field collected *A. gambiae* sample include

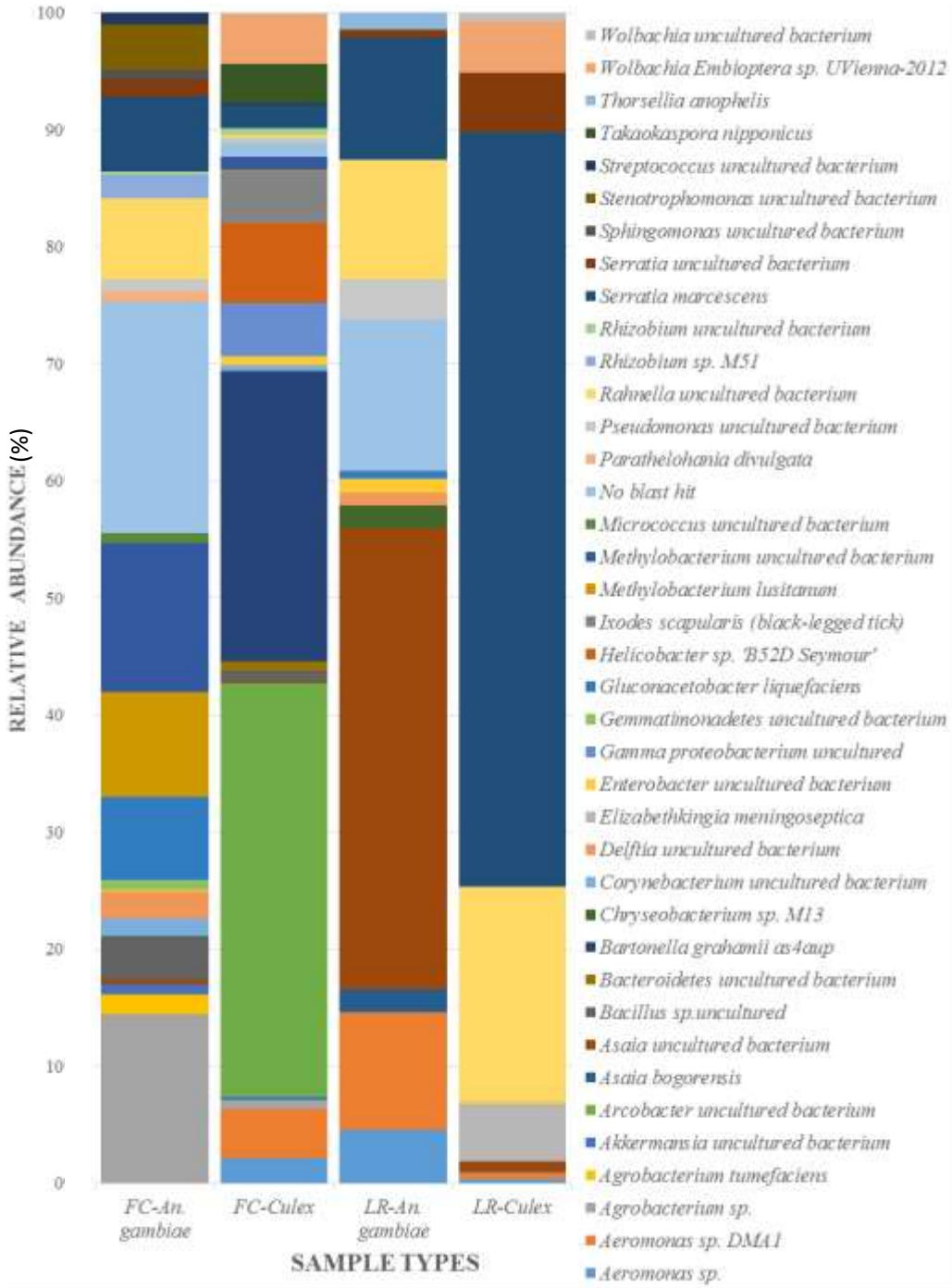

Figure 4. Relative abundance of the most predominant bacterial species from the field collected (FC) and lab reared (LR) samples.

Agrobacterium tumefaciens, *Gemmatimonadetes* uncultured bacterium, *Micrococcus* uncultured bacterium and *Rhizobium* sp. M51 (Figure 5).

Bacterial species which were recovered from all the four samples include, *Serratia marcescens*, *Asaia* uncultured bacterium, *Enterobacter* uncultured bacterium, *Pseudomonas* uncultured bacterium and *Rahnella* uncultured bacterium. *Parathelohania divulgata* and *Takaokaspora nipponicus* are fungal species recovered from the field collected *A. gambiae* and *C.quinquefasciatus* respectively (Figure 5). Detailed information on all the bacterial species recovered in this study is given in additional file 1 Table S1.

Hierarchical clustering, based on Bray-Curtis

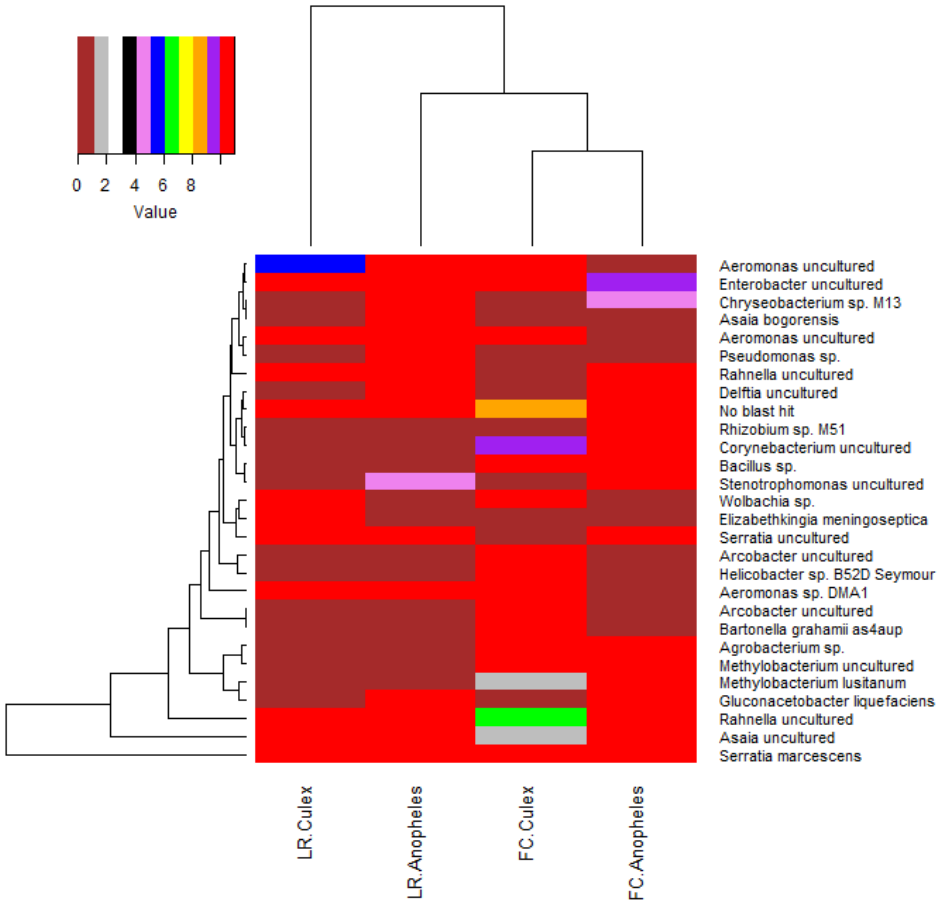

Figure 5. Hierarchical clustering of most abundant midgut bacterial species of the field collected (FC) and lab reared (LR) mosquitoes. Species level was chosen to be used in hierarchical clustering to assess the relationship between sample and taxa.

dissimilarity, showed two clusters (Figure 5). The dendogram shown on the top signify the relationship between the four samples. The bacteria composition of lab reared *A.gambiae*, field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were clustered together. Within this cluster the field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were more closely related to each other. The bacterial community recovered from the lab reared *C. quinquefasciatus* samples was observed to form a distinct cluster.

DISCUSSION

The microbes inhabiting mosquito midgut have drawn special attention in the recent past due to their interactions with both the mosquito host as well as disease causing parasites. The present study sought to investigate the composition and diversity of microbes in midguts of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes. The field collected mosquitoes showed more midgut bacterial composition and diversity than the lab reared mosquitoes. A similar observation was reported by Rani et al. (2009) in their study involving the analysis of bacterial diversity in larvae and adult midgut microflora in lab reared and field collected *Anopheles stephensi* mosquito vectors. The higher bacterial diversity in field collected mosquitoes could probably be due to the fact that wild mosquitoes are exposed to the natural environment where they feed on various natural foods that could be the source of the diverse microbes, whereas the lab reared mosquitoes are fed on artificial diet of glucose and resins. Furthermore, adult female mosquitoes require a blood meal for their egg development and the blood acquired in the field could also be a source of various bacterial flora. On the other hand, the blood given to the adult female lab reared mosquitoes is from infection-free rabbits/mice (Rani et al., 2009). In the present study, the highest number of bacterial species was detected from field collected *A. gambiae* mosquitoes followed by field collected *C. quinquefasciatus* and lab reared *A. gambiae*. The least number of bacterial species were detected from lab

reared *C. quinquefasciatus*.

Diversity indices analysis at OTU level from field collected and laboratory reared mosquitoes revealed a significant difference in microbial community composition. Field collected *A. gambiae* had the highest value of Inverse Simpson index and while the lowest was lab reared *C. quinquefasciatus* samples. The value of Inverse Simpson increases with diversity (Chandel et al., 2013). The Shannon index is another widely used index for comparing diversity between various habitats (Chandel et al., 2013). The Shannon index is a representation of both species abundance and evenness, when either of these two factors increase, the diversity index increases. Evenness was used for the estimating how well the species are evenly distributed among the samples. The lowest evenness was recorded from laboratory reared *C. quinquefasciatus* sample indicating that the bacterial species in this sample are less evenly distributed, that is, some species are more dominant than others. Comparative diversity was visualized using heatmap based on Bray-Curtis dissimilarities at species level. The microbial composition of the field collected samples at species level, were more similar compared to the laboratory reared. However, the laboratory reared samples the bacterial composition seemed to differ between *A. gambiae* and *C. quinquefasciatus*.

Members of the phylum Proteobacteria, were predominantly recovered from both the field collected and lab reared samples than those of phylum *Firmicutes*, *Actinobacteria* and *Bacteriodetes*. *Proteobacteria* were also shown to be dominant in a previous study conducted in Kenya on *A. gambiae* mosquitoes (Wang et al., 2011). Proteobacteria was the largest phylum represented by 43 species belonging to 26 genera. Some of the groups of bacteria recovered in the present study are similar to those recovered from previous culture dependent and culture-independent studies (Rani et al., 2009). Phylum *Firmicutes* consisted of ten species which were affiliated to nine genera. *Actinobacteria* represented fifteen species belonging to thirteen genera whereas *Bacteriodetes* consisted of five species belonging to five genera. Phylum *Cyanobacteria*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Archeabacteria* and *Acidobacteria* represented only a small portion of the mosquito midgut communities.

The dominant genera recovered in the present study belong to *Serratia, Asaia*, *Arcobacter*, *Rahnella*, *Bartonella*, *Aeromonas*, *Agrobacterium*, *Methylobacterium* and *Wolbachia*. The results of the study are consistent with earlier reports which have shown that that above genera are dominant (Pidiyar et al., 2004; Demaio et al., 1996; Favia et al., 2007; Dong et al., 2009). This suggests that at least a fraction of the mosquito midgut inhabitants could be common for different mosquito species inhabiting similar environments and may represent evolutionary conservation of association between bacteria and mosquito gut. Members of the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Asaia*, *Rahnella*, and *Stenotrophomonas* have been frequently reported in mosquito gut in previous studies (Pidiyar et al., 2004; Boissière et al., 2012; Chandel et al., 2013). Sequences belonging to genera *Asaia*, *Enterobacter*, *Pseudomonas*, *Rahnella* and *Serratia* were recovered from all the samples and comprise a major part of microbiota of *A. gambiae* and *C. quinquefasciatus* mosquitoes in the present study.

Serratia marcescens appeared to be the core species (23.6%) in the present study, as it was observed in both lab reared and field collected samples, suggesting that it possesses a competitive advantage over other bacterial species. *S. marcescens* is abundant in nature, and especially in the artificial foods given to the lab reared mosquitoes. Similar results were reported in five generations of lab reared *A. gambiae* (Dong et al., 2009).

Asaia uncultured bacterium species was recovered at 39.30% was more abundant in lab reared *A.gambiae*. *Asaia* has been associated with *Anopheles* species, in particular field collected *Anopheles funestus*, *Anopheles Maculipennis, Anopheles gambiae* and *Anopheles coustani*, and *Anopheles stephensi* in which *Asaia* bacteria was dominant and stably associated (Favia et al., 2007). The presence of *Asaia* species in *Anopheles* mosquito could be a target for malaria control it produces antiparasite molecules in mosquitoes that could be exploited in paratransgenic control of malaria (Damiani et al., 2010; Favia et al., 2007).

Elizabethkingia meningoseptica and *Wolbachia* sp. have repeatedly been detected in both lab reared and wild caught mosquitoes (Pumpuni et al., 1996) indicating their prevalent symbiotic association with mosquitoes. In the present study, *Wolbacha* was detected in both field collected and lab reared *C. quinquefasciatus*, previously it has been reported in several other mosquito vectors including, *Aedes*, *Coquillettida* and *Masonia* (Ricci et al., 2012).

Bacillus sp., *Stenotrophomonas*, *Micrococcus Acinetobacter*, and *Rhizobium* frequently isolated from soil and environmental samples were recovered at significantly greater numbers from the field collected mosquitoes. This suggests that the local soil and water environment plays an important role in colonization of the mosquito midgut at breeding sites or during nectar/blood feeding (Chandel et al., 2013). *Parathelohania divulgata*, *Parathelohania obesa* and *Takaokaspora nipponicus* fungal species were recovered from the field collected *A. gambiae* and *C. quinquefasciatus* but were absent in lab reared mosquitoes.

From the foregoing, the mosquito midgut has a rich diversity of symbiotic bacteria. The parasite mosquito relationship is believed to have been in existence for many years and it is likely that the acquired microflora permit the maintenance of pathogenic parasites in mosquitoes. The microbes could be benefiting the

mosquitoes by protecting them against harmful bacteria and the mosquitoes could be benefiting the parasites by lowering the mosquito immunity against the parasites.

Conclusion

The results obtained present an analysis of the composition and bacterial diversity of lab reared and wild mosquitoes using Illumina sequencing technology. The bacterial flora of adult female *A. gambiae* and *C. quinquefansciatus* midgut is diverse and is dominated by bacterial species *S. marcescens*. In future, understanding the tripartite mosquito-microbes-parasite interaction will enable us gain more insight that may be useful in the development of novel approaches for the control of malaria and other mosquito borne diseases like filariasis, dengue, Zika and Chikungunya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Additional file 1

Table S1. Midgut bacterial composition at species level and their abundance.

Species level	FC.*Anopheles*	FC.*Culex*	LR.*Anopheles*	LR.*Culex*	Total
Phylum *Archeabacteria*					
Archaea, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, *Methanobrevibacter*, uncultured archaeon	24	0	0	0	
Phylum *Acidobacteria*					
Bacteria, Acidobacteria, Acidobacteria, Subgroup 4, Unknown Family, Blastocatella, uncultured *Acidobacteria* bacterium	10	0	0	0	
Bacteria, Acidobacteria, Acidobacteria, Subgroup 6, uncultured *Acidobacteria* bacterium	32	0	0	0	
Phylum *Actinobacteria*					
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* uncultured bacterium	72	10	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, Corynebacterium, uncultured *Corynebacterium* sp.	6	6	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* unidentified marine bacterioplankton	10	3	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, uncultured, uncultured bacterium	31	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Dietziaceae, *Dietzia* uncultured bacterium	8	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Frankiales, Geodermatophilaceae, *Blastococcus* uncultured bacterium	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Cellulomonadaceae, *Actinotalea* uncultured bacterium	17	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Intrasporangiaceae, *Terrabacter* uncultured bacterium	38	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae, *Curtobacterium* uncultured bacterium	24	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Arthrobacter, *Arthrobacter* sp. TSBY-23	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Enteractinococcus, *Yaniella* sp. YUAB-SO-24	7	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Kocuria, *Kocuria* sp. oral clone AW006	11	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, *Micrococcus* uncultured bacterium	43	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae, *Nocardioides* uncultured bacterium	4	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Streptomycetales, Streptomycetaceae, Streptomyces, *Streptomyces ferralitis*	12	0	0	0	
Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, uncultured, uncultured bacterium	12	0	0	0	
Phylum *Bacteroidetes*					
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Rikenellaceae, RC9 gut group, uncultured bacterium	6	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, *Fluviicola* uncultured bacterium	14	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Chryseobacterium, *Chryseobacterium* sp. M13	5	0	113	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Elizabethkingia, *Elizabethkingia meningoseptica*	1	0	0	291	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, uncultured, uncultured Bacteroidetes bacterium	0	21	0	0	
Phylum *Chloroflexi*					
Bacteria, Chloroflexi, Thermomicrobia, JG30-KF-CM45, uncultured soil bacterium	31	0	0	0	
Bacteria, Chloroflexi, Thermomicrobia, Sphaerobacterales, Sphaerobacteraceae, Nitrolancea, uncultured *Chloroflexi* bacterium	19	0	0	0	
Phylum *Cyanobacteria*					
Bacteria, Cyanobacteria, *Chloroplast* uncultured bacterium	6	0	0	0	
Phylum *Firmicutes*					
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Anoxybacillus* uncultured bacterium	0	10	1	0	
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, Bacillus, uncultured *Bacillus* sp.	183	31	1	0	

Table S1. Contd.

Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Bacillus* uncultured bacterium	5	1	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Planococcaceae, *Lysinibacillus* uncultured bacterium	22	5	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Staphylococcaceae, *Salinicoccus* uncultured bacterium	39	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, *Atopostipes* uncultured bacterium	32	1	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, uncultured, uncultured bacterium	6	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, *Streptococcus* uncultured bacterium	53	4	0	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae, *Incertae Sedis* uncultured bacterium	26	4	1	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, uncultured, uncultured bacterium	15	0	0	0
Phylum *Gemmatimonadetes*				
Bacteria, Gemmatimonadetes, Gemmatimonadetes, AT425-EubC11 terrestrial group, uncultured bacterium	42	0	0	0
Phylum *Proteobacteria*				
Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, *Brevundimonas* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bartonellaceae, Bartonella, *Bartonella grahamii* as4aup	0	737	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, Bosea, uncultured *Bosea* sp.	37	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, *Methylobacterium lusitanum*	468	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, *Methylobacterium* uncultured bacterium	665	32	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Agrobacterium tumefaciens*	87	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. JC140	32	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. M51	106	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Agrobacterium* sp.	754	21	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, *Rhizobium* uncultured bacterium	10	18	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Paracoccus* sp.	19	1	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, *Paracoccus* uncultured bacterium	9	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Acetobacter, uncultured *Acetobacter* sp.	0	0	8	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Asaia, *Asaia bogorensis*	0	0	116	1
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	16	2	2223	55
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	0	0	47	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	5	0	39	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Gluconacetobacter, *Gluconacetobacter liquefaciens*	370	0	37	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* Embioptera sp. UVienna-2012	0	125	0	261
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* uncultured bacterium	0	5	0	43
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, mitochondria, *Triticum aestivum*	17	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Rickettsiaceae, Rickettsia, uncultured Rickettsia sp.	19	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomona*s uncultured bacterium	21	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*, uncultured *Firmicutes* bacterium	24	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, *Delftia* uncultured bacterium	74	0	59	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Delftia, uncultured *Delftia* sp.	46	0	7	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae, Oxalicibacterium, *Oxalicibacterium flavum*	30	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Neisseriales, Neisseriaceae, uncultured, uncultured bacterium	7	1	0	0

Table S1. Contd.

Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Campylobacteraceae, *Arcobacter* uncultured bacterium	0	1050	0	0
Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter, *Helicobacter* sp. 'B52D Seymour'	0	204	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, *Aeromonas* sp. DMA1	0	125	588	37
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, uncultured *Aeromonas* sp.	0	0	12	2
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, *Aeromonas* uncultured bacterium	0	64	254	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Enterobacter* uncultured bacterium	10	23	71	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Escherichia-Shigella, *Serratia marcescens*	0	0	1	4
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	364	7	399	1050
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	207	0	210	31
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	168	48	365	2146
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	157	14	232	1236
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	0	0	15	98
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	11	3	69	399
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	1	0	7	23
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia* sp. DR.Y5	0	0	1	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	4	0	4	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	28	0	0	1
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	42	0	12	59
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	0	0	11	91
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	19	0	10	53
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Thorsellia, *Thorsellia anophelis*	0	0	81	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	69	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	65	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter, *Acinetobacter* sp. B7_2TCO2	9	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	3	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	1	1	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Enhydrobacter* uncultured proteobacterium	13	4	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Psychrobacter* uncultured bacterium	11	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	21	14	26	6
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	28	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured Pseudomonas sp.	1	0	177	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas* uncultured bacterium	196	0	5	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas*, uncultured bacterium	15	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadales Incertae Sedis, *Steroidobacter* uncultured bacterium	16	0	0	0
Phylum *Spirochaetae*				
Bacteria,Spirochaetae,Spirochaetes,Spirochaetales,Spirochaetaceae,uncultured,*Spironema culicis*	0	6	0	0
Phylum *Verrucomicrobia*				
Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	47	0	0	0

Table S1. Contd.

Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	0	7	0	0	
Phylum *Eukaryota*					
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, Parathelohania, *Parathelohania divulgata*	51	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Parathelohania Parathelohania obesa*	33	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Takaokaspora Takaokaspora* nipponicus	0	100	0	0	
No blast hit	1030	34	757	0	1721
Total abundance	6214	2890	5960	5949	21666