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Dynamics of gut microbiota and their hemolytic activity in vector and non-vector mosquitoes before and after feeding

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Abstract

Background The mosquito midgut serves as a critical interface where the resident microbiota interacts with acquired pathogens following a blood meal. While some species are known to antagonize the others facilitate the multiplication and establishment of pathogens. Despite this critical interface, our understanding of the midgut microbial landscape remains incomplete. This study aims to characterize the dynamics of the gut microbiota in both unfed and post-fed states across major vector and non-vector species, with a particular focus on the prevalence of haemolytic bacterial communities.

Methods Both culture-dependent and independent methods were used to determine the diversity of the gut bacterial community pre- and post-feeding. The haemolytic pattern of the culturable bacteria was determined in blood agar plates. The species richness and Shannon alpha diversity of the bacterial communities pre and post feeding were determined.

Results Analysis of the haemolytic pattern showed that both haematophagous and non-haematophagous bacterial isolates were in equal proportion in unfed conditions, non-haemolytic biota dominate haematophagous mosquitoes while haemolytic communities dominate in non-hematophagous mosquitoes after feeding. The unweighted UniFrac PCoA analysis showed that feeding exerted fewer changes in the richness of the gut bacterial community of the tested mosquitoes, except *Armigeres subalbatus*. The weighted UniFrac PCoA analysis showed that the gut bacterial community of unfed mosquito species was clustered together, except *Aedes aegypti*, while after feeding, a huge change in the relative abundance was observed in *Ar. subalbatus*. Although the phylum *Proteobacteria, Acidobacteria, Planctomycetes, Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Crenarchaeota*, and *Chloroflexi* play a dominant role and have changed considerably after feeding, a majority of the bacterial residents are present in extremely small numbers as minor resident biota. A comparative analysis showed that the species richness of the gut bacterial community increases after feeding, while the Shannon diversity showed no change between pre and post feeding.

Conclusion The present study provides a comprehensive overview of the gut bacterial community in hematophagous and non-hematophagous mosquitoes pre and post feeding. While the blood meal serves as a primary driver of taxonomic shifts, the response of haemolytic bacteria is species-specific. In general, post-feeding supports haemolytic bacteria in non-hematophagous and non-haemolytic bacteria in hematophagous mosquitoes. While feeding caused a shift in the species richness of the gut bacterial community in *Ar. subalbatus* and the relative abundance of gut biota changed in both *Ae. aegypti* and *Ar. subalbatus*, the changes were minimal in the rest of the species.

Introduction

Representing over 3,500 described species, mosquitoes are globally distributed and highly adapted insects that thrive in various environments. While they play relevant ecological roles, a subset of several hundred species remains a primary threat to public health [1]. Anopheles, Aedes, and Culex are three major genera of vector mosquitoes that transmit malaria, dengue, chikungunya, Zika, yellow fever,

Japanese encephalitis, West Nile fever, and lymphatic filariasis [2]. More than half of the global human population is at risk of one or other diseases transmitted by mosquitoes, causing huge morbidity and mortality [3]. The World Malaria Report 2025 estimates 282 million cases and 610 000 deaths in 80 malaria-endemic countries in 2024, with an increase of 9 million cases compared with 273 million cases in 2023 [4]. Global dengue incidence has escalated substantially over the last five years, culminating in a record 14.1 million reported cases in 2024. This figure represents a twofold increase over the 7 million cases documented in 2023, underscoring an accelerating public health crisis [5]. Another arboviral disease, chikungunya, is also emerging and re-emerging in many parts of the world, with a staggering half million reported cases between January 1 and September 30, 2025 [6]. This is followed by Yellow fever with an estimated 67 000–173 000 severe infections and 31 000–82 000 deaths, Japanese encephalitis with 100 000 cases and 25 000 deaths. Around 25 million men were affected by hydrocele due to lymphatic filariasis, and 15 million people had lymphoedema [7]. While the threat of mosquito-borne diseases is growing continuously, our current countermeasures are largely inadequate [8].

After being acquired through a blood meal, mosquito-borne pathogens (parasites or arboviruses) must interact with the gut microbiota of the mosquito and successfully establish an infection to render the mosquito competent for disease transmission [9]. Members of the mosquito gut microbiota can produce factors that directly inhibit or enhance the establishment of pathogens. For example, *Chromobacterium* sp. isolated from the midgut of *Aedes aegypti*, secretes a protease known as aminopeptidase which synergistically with a co-expressed neutral protease degrades the viral envelope (E) protein. By destabilizing the virion, it disrupts the integrity of the dengue virus particle, that prevents its attachment to and subsequent infection of gut epithelial cells [10]. Furthermore, *Chromobacterium* sp. produces romidepsin, a potent histone deacetylase (HDAC) inhibitor that functions as an anti-plasmodial agent. This molecule effectively inhibits the development of *Plasmodium* parasites within the mosquito midgut by suppressing their HDAC activity, thereby impairing both asexual and sexual stages (including gametocytes and ookinetes) and blocking further transmission [11]. One of the midgut microbiota, *Serratia ureilytica* of *Anopheles stephensi* and *A. gambiae*, produces specific effectors such as AmLip, a lipase that directly and selectively kills gametocytes, inhibiting *Plasmodium falciparum* and *P. berghei* ookinete [12]. A symbiotic bacterium, *Rosenbergiella* YN46, resists flavivirus infection by acidifying the gut lumen of *Aedes albopictus* and inactivating the viral particle [13]. *Serratia marcescens*, a gut microbiota of *Anopheles stephensi*, activates the immune system of the mosquitoes by upregulating 33 immunity genes, including those encoding anti-Plasmodium factors, and prevents the development of Plasmodium parasites in the mid gut [14]. *Proteus sp*. and *Paenibacillus sp*. in the midgut of *A. aegypti* upregulate several antimicrobial peptide genes and decrease DENV-2 establishment in the midgut [15]. In contrast, members of the gut microbiota also encourage the development of pathogens. For example, some members of the family Enterobacteriaceae enhance the development of *Plasmodium* parasites in the midgut of *A. gambiae* and promote malaria transmission [16]. The gut microbiota in *A. gambiae* elevates the level of AMP Cecropin, which promotes infection of the midgut by the alpha virus o'nyong-nyong (ONNV) [17]. *Serratia odorifera*, found in *Ae. aegypti*, enhance the replication of dengue virus (DENV) and Chikungunya virus (CHIKV). This occurs through the interaction of its P40 protein with

prohibition on the mosquito midgut membrane, which suppresses the host's immune response [18]. *Asaia bogorensis* causes an increase in pH and biochemical change after glucose feeding in the mid-gut of *An. stephensi*, which promotes the sexual development of malaria parasites [19].

Thus, the bacteria in the midgut of mosquitoes play an intricate role in the establishment of infectious microbes and transmission of diseases. Consequently, understanding the bacterial biota in the midgut before and after blood feeding may assist in the development of novel methods to reduce disease transmission. Although previous studies reported an increase in gut microbiota after blood feeding [20, 21], the exact changes in gut microbiota before and after blood feeding and the existence of hemolytic bacterial biota among the major mosquito vectors are not clearly defined. Since anautogenous mosquitoes require animal blood for vitellogenesis, investigating the prevalence of hemolytic bacteria within the midgut is essential for understanding their role in nutrient acquisition and reproductive fitness. Although it has been reported that different blood meal source may influence the gut microbiota differently [22, 23] and the antibiotic treatment affects RBC digestion in the midgut of *Ae. aegypti* [21], the influence of blood meal on overall bacterial biota in major hematophagous and non-hematophagous mosquitoes with special reference to the hemolytic bacteria has not been explored yet. Moreover, identification of optimal microbial assembly is a crucial step in developing microbial consortia for various biotechnological applications, including vector control [24].

Therefore, it is important to analyze the dynamics of the midgut bacterial biota—with special reference to hemolytic bacteria—in major hematophagous mosquitoes both before and after blood feeding, and compare these results with the non-hematophagous *Toxorhynchites splendens.* This analysis will highlight how blood meals influence gut bacterial diversity across both groups. Accordingly, the present study employs culture-independent methods to characterize the midgut microbiota dynamics of medically significant hematophagous vectors—*Culex tritaeniorhynchus*, *Cx. quinquefasciatus*, *Aedes aegypti*, *Anopheles stephensi*, and *Armigeres subalbatus*—as well as the non-hematophagous predator, Toxorhynchites splendens. Additionally, culture-dependent methods were used to determine the distribution of hemolytic and non-hemolytic bacteria in all aforementioned species, with the exception of *Ar. subalbatus*.

Materials and Methods

Mosquito collection and rearing

Adult female mosquitoes from five vector species - *Cx. tritaeniorhynchus, Cx. quinquefasciatus, Ae. aegypti, An. stephensi*, and *Ar. subalbatus* were collected from in and around Madurai (latitude: 9°56'10.97" N, longitude: 78°08'09.33" E; elevation: 140 m) in Tamil Nadu State, India, by indoor resting collection. The adult *Tx. splendens* were collected from the tree holes of the Alagarkovil hills (latitude: 10°04’56.29” N, longitude: 78°13’01.97” E, elevation: 349 m) in the Madurai district of Tamil Nadu state, India. The collected mosquitoes were stabilized in the mosquito colony of this centre (Indian Council of Medical Research-Vector Control Research Centre [ICMR-VCRC] Field Station, Madurai, Tamil Nadu,

India) for three generations before being used in the experiment. The standard operating procedure as described by Zheng et al. [25] was followed with slight modification for the mass rearing, storage, and hatching of all mosquito species except *Ar. subalbatus* and *Tx. splendens*. Because their growing conditions and feeding preferences are different, *Ar. subalbatus* and *Tx. splendens* were reared according to the methodologies described by Yang et al. [26] and Schiller et al. [27], respectively, with local modifications. In general, adult mosquitoes of all the species were maintained in 60 × 60 × 60 cm cages (BugDorm-6610, India) at room temperature (RT) of 27 ± 1°C and relative humidity (RH) of 85 ± 5% with a photoperiod of 12:12 h (L:D). A cotton pad soaked in 10% glucose, along with wet raisins, was given in each cage as a sugar source for the adult mosquitoes. The glucose pad and wet raisins were changed at an interval of 2 days to avoid fungal growth. After 2 days of glucose feeding, the female hematophagous mosquitoes were allowed to take a chicken blood meal using an artificial blood feeding apparatus, and then were allowed to rest for 3 days before being placed for oviposition. A filter paper (Whatman) strip was kept half-soaked in each half-water-filled bowl for oviposition [28]. In addition to the raisins, cotton pads soaked in honey were kept in the cages of *Tx. splendens* as their feeding source as described [27].

Mosquito selection, midgut dissection, and inoculation in culture media

For the experiment purpose, a separate cage for each species was maintained with homogenous female adults that emerged almost within an hour. The females were allowed to take sterile sugar solutions (10% glucose) ad libitum in the cage for one day. Then, from each species, 10 females were taken from the cage and were cold anesthetized. Then the mosquitoes were surface sterilized to remove surface bacteria by rinsing in 2% bleach solution, a 5-min rinse in 70% ethanol, followed by 5-minute rinses in 1× sterile phosphate buffered saline (PBS). Aliquots of 100μL from the last washes were plated in nutrient agar as a control for the surface sterilization process. The mosquitoes were placed on a double cavity slide containing a drop of sterile PBS and dissected under a stereomicroscope. By taking utmost care, the midgut was removed from the abdomen and transferred into a 1.5 ml Eppendorf tube containing 200 μl of sterile PBS. Ten midgut samples from each mosquito species (either unfed [UF] or after feeding and egg laying [AF]) were pooled into one, and the content of the tube was thoroughly mixed using a pellet pestle (Sigma-Aldrich).

Both culture-dependent and culture-independent techniques have been used to explore the mosquito microbiota. A total of 12 midgut pools were prepared to cover all six species of unfed and after feeding groups (6 UF and 6 AF). While 100 μl from each tube was preserved for the metagenomic analysis of the bacterial biota, the remaining 100 μl was serially diluted (10^{-1} through 10^{-7}), and an aliquot of 100 μl was inoculated in Brain Heart Infusion agar plates (HiMedia, India) by the spread plate method (except the sample from *Ar. subabatus*). The inoculated BHI agar plates were incubated at 28°C for 48 h and then transferred to 4°C. Based on the colony morphology (colour, size, shape, opacity, margin, elevation, and viscosity), Gram staining, and motility, the isolates were separated and pure cultured in Blood agar

plates (HiMedia, India) supplemented with 10% defibrinated sheep blood. The haemolytic pattern of each isolate was recorded. The entire microbiological process was performed in a sterile environment by strictly following aseptic procedures and negative controls (sterile PBS, culture plates). All the materials required for the dissection of mosquitoes were autoclaved at 121°C for 20 min. Other materials, such as a stereomicroscope, pipettes, and work space, were sanitized with 70% ethanol [29]. The diversity of haemolytic and non-haemolytic bacterial isolates in unfed and after feeding mosquitoes was tabled and presented through a bar graph and a pie-chart.

Genomic DNA Isolation and PCR amplification of 16S rRNA gene from bacterial isolates (Culture-dependent method)

Genomic DNA was extracted from bacterial cultures using the NucleoSpin® Tissue Kit, followed by 16S rRNA gene amplification (~ 1.5 kb) using primers 16S-RS-F (5'- CAGGCCTAACACATGCAAGTC-3') and 16S-RS-R (5'- GGGCGGWGTGTACAAGGC-3') on a GeneAmp PCR System 9700. Purified PCR products were sequenced via the Sanger method on an ABI 3500 DNA Analyzer, with sequence analysis and alignment performed using Sequence Scanner Software v1 and Geneious Pro v5.1. The sequences obtained were compared with the NCBI GeneBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) and the EzTaxon server (http://www.ezbiocloud.net/eztaxon) to search for homologous sequences [30, 31].

Phylogenetic analysis of gut bacterial isolates

The phylogenetic relationships of the isolated bacterial community were inferred using the Maximum Likelihood (ML) method based on the Tamura-Nei model [32]. The tree with the highest log-likelihood was selected for the final representation [33]. To evaluate branch support, a bootstrap test was performed, and the percentage of replicate trees in which the associated taxa clustered together is shown next to the branches.

The initial tree for the heuristic search was automatically selected by comparing the log-likelihood scores of a Neighbor-Joining (NJ) tree and a BioNJ tree, choosing the topology with the superior value [34]. Evolutionary analyses, including the calculation of pairwise distances and the heuristic search, were conducted in MEGA 11, utilizing the Kimura 2-Parameter model (K2P) gamma distribution sites (K2P + G) [35]. In the phylogenetic tree, the haemolytic bacterial isolates are indicated by red circle, while non-haemolytic isolates are represented by green triangles.

DNA extraction and 16S rRNA gene amplification of pooled midgut samples (culture-independent method)

Metagenomic DNA was extracted from pooled midgut samples using the DNeasy Blood and Tissue Kit (Qiagen, USA) in strict accordance with the manufacturer's guidelines. Following isolation, a NanoDrop

spectrophotometer (Thermo Scientific) was used to assess the concentration and purity of the resulting DNA. Then, A ~ 1.5 kb segment of the 16S rRNA gene was targeted using the specific primer pair 16SRS-F (5'-GCCTACGGGNGGCWGCAG-3') and 16S-RS-R (5'-ACTACHVGGGTATCTAATCC-3') for amplification. The amplicon libraries were constructed utilizing the Nextera XT Index Kit (Illumina Inc.), adhering to the established 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B of Illumina inc.,). Each 25 µl PCR reaction mixture contained 12.5 µl of 2× KAPA HiFi HotStart ReadyMix, 5 µl of each primer (1 µM), and 2.5 µl of DNA template adjusted to 5 ng/µl. The PCR amplification involved an initial 3-minute denaturation at 95°C, followed by 35 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s, concluding with a 5-minute final extension at 72°C. To confirm successful amplification, 3 µl of the PCR product was visualized on a 1.2% agarose gel, run at 120V for roughly 60 minutes until sufficient migration was achieved.

Library preparation and High-throughput sequencing

The amplicons passed quality control with Illumina adaptor and were amplified using i5 and i7 primers. This step incorporated multiplexing indices and the P5/P7 adapters necessary for cluster generation, following the standard Illumina sequencing protocol. The resulting libraries were purified with Beckman Coulter AMPure XP beads and their concentrations determined via Qubit Fluorometry (Invitrogen). To assess library integrity, the samples were processed on a 4200 TapeStation (Agilent Technologies) using D1000 ScreenTape. Based on the mean peak sizes identified, libraries were diluted to a final concentration of 10–20 pM and loaded onto the Illumina MiSeq platform. The sequencing utilized a paired-end approach, where reagents facilitated sample binding to adapter oligos on the flow cell. This configuration allowed selective cleavage of forward strands after reverse strand synthesis, enabling template fragments to be sequenced from both directions.

Bioinformatic Analysis

To refine the raw data, high-quality reads were generated using Trimmomatic v0.38 by stripping adapter sequences and filtering out ambiguous reads (over 5% "N" bases) or low-quality sequences (more than 10% of bases with a Phred score < 20). This processing utilized a 20 bp sliding window and maintained a 100 bp minimum length. Subsequently, FLASH (v1.2.11) was employed to merge paired-end reads into single-end sequences. Bioinformatic analysis was conducted via QIIME, where Operational Taxonomic Units (OTUs) were clustered by similarity and referenced against the Greengenes database (v13_8) to assign taxonomy. The composition of gut bacterial community was evaluated through several metrics (a) Alpha Diversity: Species richness and evenness were quantified using Shannon index plots and rarefaction curve analysis, (b)Beta Diversity: Variations between samples were visualized using weighted and unweighted PCoA plots, (c)Taxonomic Visualization: A heatmap was constructed to illustrate the relative abundance of OTUs across the different samples [36–40].

Results

Diversity of the Midgut Microbiota - Culture-dependent analysis:

A total of 29 bacterial isolates from 21 species of gut bacterial communities were isolated from either unfed (UF) or post-fed (AF) adult mosquitoes belonging to *Cx. tritaeniorhynchus, Cx. quinquefasciatus, Ae. aegypti, An. stephensi*, and *Tx. splendens*.

Haemolytic pattern shifts

Analysis of the haemolytic pattern of the bacterial colonies on blood agar showed significant community restructuring after feeding and oviposition (Table 1). The shifts in haemolytic and non-haemolytic bacterial communities after feeding were unique in each mosquito species. While both haemolytic and non-haemolytic biota increased after feeding in *Ae. aegypti*, the haemolytic and non-haemolytic biota vanished in *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* respectively. In *An. stephensi*, the number of haemolytic bacteria reduced and non-haemolytic biota appeared newly after feeding (Fig. 1). While unfed mosquitoes harbour both or either non-haemolytic and haemolytic bacterial taxa, feeding - whether on blood or honey - differentially impacted the abundance of haemolytic and non-haemolytic biota. This shift in bacterial taxa across mosquito species indicates a feeding-associated selection/enrichment for both haemolytic and non-haemolytic phenotypes.

The comparative pie-chart analysis of both haemolytic and non-haemolytic microbiota in unfed and in after-feeding haematophagous and non-haematophagous mosquitoes clearly showed the changing dynamics of the community after feeding. While the haemolytic bacteria have increased by 17% after feeding in non-haematophagous mosquitoes, the haematophagous mosquitoes showed a 4% reduction after feeding (Fig. 2). Whereas a slight increase of non-haemolytic bacteria (from 60.5% to 62.9%) was observed in haematophagous mosquitoes, a drastic reduction (42.9% to 25%) of the same was observed in non-haematophagous mosquitoes. Although it varies among mosquito species, in general, the proportion of change in haemolytic and non-haemolytic bacterial biota was less in haematophagous and more in non-haematophagous mosquitoes. Overall, non-haemolytic bacteria dominate hematophagous mosquitoes, and haemolytic communities dominate in their non-hematophagous counterparts after feeding.

Table 1. Hemolytic patterns of bacteria isolated from the midgut samples of both vector and non-vector mosquitoes. UF: unfed; AF: after feeding and egg laying.



Sl. No.	Name of the Bacteria	NCBI Gene bank Accession No.	*Aedes aegypti*		*Culex tritaeniorhynchu*		*Culex quinquefasciatus*		*Anopheles stephensi*		*Toxorhynchites splendens*	
			UF	AF	UF	AF	UF	AF	UF	AF	UF	AF
1	*Bacillus altitudins*	MW629907										
2	*Bacillus cereus* A	MW629909										
3	*Bacillus cereus* B	MW629911										
4	*Bacillus cereus* C	MW629913										
5	*Bacillus paramycoides*	MW629910										
6	*Brevibacterium casei*	MW629899										
7	*Brevundimonas faecalis*	MW629917										
8	*Chryseobacterium candidae*	MW629895										
9	*Chryseobacterium flavum*	MW629939										
10	*Elizabethkingia anophelis* A	MW629905										
11	*Elizabethkingia anophelis* B	MW629931										
12	*Elizabethkingia anophelis* C	MW629933										
13	*Elizabethkingia anophelis* D	MW629935										
14	*Enterobacter cloacae*	MW629884										
15	*Enterobacter hormaechei*	MW629881										
16	*Enterobacter mori*	MW629897										
17	*Enterobacter roggenkamppii*	MW629937										
18	*Enterococcus faecalis*	MW629891										
19	*Klebsiella pneumoniae*	MW629889										
20	*Klebsiella quasipneumoniae*	MW629890										
21	*Luteimonas yindakuii*	MW629928										
22	*Lysinibacillus fusiformis* A	MW629887										
23	*Lysinibacillus fusiformis* B	MW629929										
24	*Micrococcus luteus* A	MW629919										
25	*Micrococcus luteus* B	MW629893										
26	*Staphylococcus gallinarum*	MW629915										
27	*Staphylococcus haemolyticus*	MW629901										
28	*Stutzerimonas stutzeri* A	MW629885										
29	*Stutzerimonas stutzeri* B	MW629923										
	H/Non-H		1/2	2/3	2/0	0/3	0/4	2/0	3/0	2/1	1/1	2/1

Taxonomic Dominance

In the unfed (UF) state, the midgut was dominated by a set of genera, mainly *Elizabethkingia, Bacillus, Luteimonas*. Following a meal, the community shifted toward dominance by *Enterobacter* and *Chryseobacterium*. Members of the genera *Bacillus, Brevibacterium, Elizabethkingia, Klebsiella, Lysinibacillus*, *Micrococcus, Staphylococcus*, and *Stutzerimonas* were present in both the unfed and post-feeding stage among the mosquito species (Table 1).

Taxonomic relationships

The phylogenetic analysis illustrates the evolutionary relationships of the gut bacterial isolates from haematophagous (panel A) and non-haematophagous (panel B) mosquitoes (Fig. 3). The taxonomic positions of haemolytic (HL) and non-haemolytic (NHL) bacteria reveal distinct clustering patterns based on both the functional traits in both haematophagous and non-haematophagous mosquitoes.

Metabolites/enzymes production:

An extensive literature survey revealed that bacterial species isolated from the midgut of mosquito species in the present study can produce several active metabolites, enzymes (Fig. 4), which could play a crucial role in the survival of mosquito species.

Diversity and Composition of the Mosquito Midgut Microbiota: Culture-independent analysis:

Metagenomic analysis of the midgut bacterial taxa in six vector species - *Cx. tritaeniorhynchus*, *Cx. quinquefasciatus*, *Ar. subalbatus*, *Ae. aegypti*, and *An. stephensi*—and one non-vector species, *Tx. splendens*, revealed a complex bacterial community at the genus level across all species. Furthermore, this microbial complexity shifted significantly between unfed (UF) and post-fed (AF) conditions

Species richness of the gut bacterial community

The unweighted UniFrac PCoA analysis based on the presence-absence of taxa revealed patterns of variation in the gut bacterial community associated with feeding status and mosquito species (Fig. 5). The unfed mosquito samples (marked as blue dots) largely clustered together, showing a substantial overlap of bacterial communities. The post-feeding samples (marked as red dots) were mostly positioned to their corresponding unfed samples, indicating that most bacterial lineages detected prior to feeding were retained after feeding and egg laying. The principal coordinate axes explained a moderate proportion of the total variation (PC1: 17.68%, PC2: 10.42%, PC3: 8.87%). Compared with the weighted UniFrac analysis, which is described later in this section, the separation between unfed and post-feeding samples along the axes was limited in this unweighted UniFrac analysis, indicating that feeding had relatively less effect on the overall richness of the gut microbiota. However, the richness of the bacterial community in one mosquito species, *Ar. subalbatus*, exhibited a marked separation along PC1, with its unfed and post-feeding samples positioned away from the main cluster (3→11), showing a total shift in the richness of the bacterial community after feeding.

Rarefaction analysis based on OTUs which was done to assess the sequencing depth adequacy and to compare microbial richness between unfed (UF) and after feeding and egg laying (AF) samples across all mosquito species (Fig. 6). Rarefaction curve for the entire dataset indicated that except for sample No.3 (unfed *Ar. subalbatus*), the rest of the samples, the rarefaction curves showed a steep initial rise followed by a gradual approach towards saturation, indicating that the sequencing depth was sufficient to capture the majority of bacterial diversity in each of the samples. Although the convergence of curves toward a plateau in both UF and AF groups indicates that both groups are rich in bacterial biota, the difference between the groups is not clearly visible, as the curves overlap in many places. The

overlapping of curves showed that the difference in the richness between them seems to be less. Reads per midgut pool of mosquito samples gave 2,435 to 4,640 OTU (Table 2).

Table 2. Changes in species richness and Shannon alpha diversity of the gut bacterial community based on metagenomic analysis.

	Species	Sample No	Feeding status	OTU	Change in species richness	Shannon alpha diversity	Change in Shannon alpha diversity
1	*Culex tritaeniorhynchus*	1	UF	4528	84	10.1	0 --
		9	AF	4612		10.1	
2	*Culex quinquefasciatus*	2	UF	4360	55	9.9	0.2
		8	AF	4415		10.1	
3	*Armigeres subalbatus*	3	UF	2435	1452	8.1	0.2
		11	AF	3887		7.9	
4	*Aedes aegypti*	4	UF	4553	61	8.3	1.7
		12	AF	4492		10	
5	*Anopheles stephensi*	5	UF	4311	110	10	0 --
		7	AF	4201		10	
6	*Toxorhynchites splendens*	6	UF	4251	389	9.3	0.2
		10	AF	4640		9.5	

Relative abundance of the gut bacterial community

Weighted UniFrac PCoA plot was drawn to determine the relative abundance of the gut microbiome in the midgut of selected mosquito species, during unfed and post fed status (Fig. 7). The analysis revealed that, except for *Ae. aegypti* (No. 4), the gut microbial communities in all other unfed mosquito species were clustered closely, suggesting a conserved baseline gut microbiome. In contrast, post-feeding samples from each species shifted in distinct directions, indicating species-specific restructuring of microbiota after feeding and egg laying. The magnitude of displacement varied among species, reflecting the varied responsiveness of the microbiome, likely driven by changes in the dominant bacterial taxa. While two species, *Ar. subalbatus* (3→11) and *Ae. aegypti* (4→12) have shown a clear shift in the relative abundance, the microbial communities in other species clustered close before and after feeding. This suggests that while gut bacterial communities are conserved with limited but notable restructuring in some mosquito species, feeding caused a total shift in the gut bacterial communities in others

Bar plot comparison clearly demonstrates the feeding-related shift in the relative abundance of the bacterial community (Fig. 8). Although a marked change in the composition of microbiota was observed following blood feeding and egg laying (AF) in all mosquito species, the changes were prominent in *Ar. subalbatus* and *Ae. aegypti*.

Heatmap Analysis presents the relative abundance of different bacterial biota in the gut of mosquito species. The analysis of the heat map revealed a profound shift in the gut microbiota after a blood or honey meal (Fig. 9). Among the species, the shift was prominent in *Ar. subalbatus* followed by *Ae. aegypti* and *Tx. Splendens*. Overall, bacteria from the phylum *Proteobacteria, Acidobacteria, Planctomycetes, Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Crenarchaeota, and Chloroflexi* play a dominant role and have changed considerably after feeding. Relatively, a large proportion of the map does not show any change in colour, indicating that those phyla starting from *Gemmatimonadetes* to *Fusobacteria* in the heat map are minor components of the overall microbial community. Those phyla, although they exist, their proportion relative to the dominant phyla, such as Proteobacteria, is negligible and close to zero. While the heatmap represents the presence of diverse microbial phyla, it highlights the changes in the most dominant and dynamic members of the community. Our results show that a concentration of biological activity in just a few phyla, while the majority of potential bacterial residents are either present in extremely small number or unable to thrive in the gut environment. Those minor resident biotas might play a significant role in the mosquito gut, regardless of whether the mosquito is fed or unfed. These minor resident biotas may fulfil essential functions across both sugar-fed and blood-fed physiological states.

Species richness and Shannon diversity

The alpha diversity of the mosquito midgut microbiota was altered in response to a blood meal and subsequent egg laying (Fig. 10). Across all mosquito species investigated (Cx. tri, Cx. qui, An. ste, Tx. spl, Ae. aeg, and Ar. sub), the mean species richness based on OTUs was notably lower in unfed individuals compared to those sampled after feeding and egg laying. More specifically, the species richness of the gut bacterial community has drastically increased after feeding in *Ar. subalbatus*.

In contrast, the Shannon alpha diversity index, which incorporates both richness and evenness, displayed a drastic increase in the relative abundance of the gut bacterial community in *Ae. aegypti*, the rest of the mosquito species showed only minor change in the relative abundance of gut bacterial community. The trendlines also showed an overall increasing species richness from UF to AF; the Shannon alpha diversity did not show any change between UF and AF. Specifically, while species richness drastically increased after feeding in Ar. sub, (1452 OTU), followed by Tx. spl (389 OTU), Cx. tri (84 OTU) and Cu. qui (55 OTU), the same has declined in Ae. aeg (61 OTU) and An. ste (110 OTU). Conversely, the Shannon diversity index exhibited a decrease only in Ae. aeg (0.2), the rest of the species have shown either no

change (Cu. tri and An. ste) or an increase (Cu. qui – 0.2; Ae. aeg – 1.7 and Tx. spl – 0.2) (Table 2). This suggests that the total number of species increased in most species after feeding, the evenness of the community structure either increased (in Cu. qui, Ae. aeg., and Tx. spl), no change (Cu. tri and An. ste), or decreased (Ar. sub). This is likely due to a few specific bacterial taxa becoming highly abundant and dominating the gut environment after the blood meal while the others becomes recessive.

Discussion

The mosquito gut microbiota has been shown to be a potential source for developing novel combat strategies to fight mosquito-borne diseases by rendering mosquito vectors refractory to arthropod-borne human pathogens [41]. Despite growing interest in engineering the mosquito microbiome for vector control, our understanding of these microbial ecosystems remains in its infancy, leaving vast functional landscapes yet to be explored. This research presents a comprehensive comparative analysis of taxonomic shifts within the gut microbiota of vector and non-vector mosquitoes in response to feeding, integrating culture-dependent and culture-independent techniques. To our knowledge, this is the first study to compare the gut bacterial profiles of major vector and non-vector mosquitoes, specifically focusing on the prevalence of haemolytic bacteria.

Impact of Blood Ingestion on Microbiota Dynamics

In hematophagous insects, the vector competence is intrinsically linked to their blood-feeding habits [42]. Ingesting a blood meal, usually 2–3 times their bodyweight, exerts a transformative effect on the gut bacterial community [43]. During this process, the pathogen has to encounter the gut microbiota and overcome this critical bottleneck to infect the vector. The community assembly of these microbes can modulate pathogen survival directly (e.g., through lysis or biofilm formation) or indirectly by priming the immune system of the vector against the pathogen [44]. Our findings delineate the shifts in both culturable and non-culturable gut microbiota across vector and non-vector mosquitoes, offering a detailed comparison of community structure in response to feeding. A key focus was haemolysis, a major bacterial virulence factor associated with blood meal digestion. Our study shows an increase in haemolytic bacteria after a blood meal in *Ae. aegypti* which is consistent with the report of Gaio Ade et al. (2011) that the synergistic involvement of haemolytic bacteria in haemolysis contributes to protein digestion in *Ae. aegypti*. However, for the rest of the haematophagous mosquito species, our study presents a different picture of either maintaining or decreasing haemolytic bacterial community after feeding. In contrast, the haemolytic bacteria have increased substantially (by 17%) after honey feeding in the non-haematophagous mosquito, *Tx. splendens*. The reduction of haemolytic bacteria after a blood meal in haematophagous species suggests that the blood meal seems to be antagonistic on haemolytic gut bacterial community. While the release of reactive oxygen species (ROS) by gut epithelial cells was antagonistic to the gut bacterial community, the release of haem after a blood meal reduces the ROS and favours the proliferation of gut bacteria [43]. Although our report is consistent with Gaio Ade et al. (2011) that haemolytic bacteria often play an essential role in enhancing the digestion of blood, we further clearly demonstrate their role in the digestion of even non-blood-based meal.

Diversity and Community Assembly

While the larval gut microbiome of *Toxorhynchites amboinensis* is already documented [45], the present study characterizes the adult *Tx. splendens* gut microbiota, distinguishing between haemolytic and non-haemolytic strains. Our data suggest that post-feeding shifts primarily stem from the selective suppression or dominance of specific taxa. The sustained dominance of hemolytic bacteria in *Tx. splendens* compared to its suppression in hematophagous mosquitoes suggests that these communities may be physiologically discouraged in the latter.

Beyond dominant taxa, our heatmap analysis revealed a "rare biosphere" of low-abundance species. Though often overlooked, these species serve as reservoirs of genetic diversity that contribute to the host's overall fitness [46–47]. While Salgado *et al.* (2024) reported a reduction in microbial diversity after blood feeding in *Anopheles* [48], we observed a similar reduction in diversity across all tested species, regardless of whether they consumed blood or honey. This shift highlights the dynamic nature of the mosquito gut as it adapts to the physiological stress of the host diet, impacting immune response and nutrient acquisition [49].

Challenging Established Paradigms

Contrary to the general belief that blood meals significantly increase gut bacterial load [50], the present study observed only a slight increase in species richness and relatively stable abundance compared with the gut bacterial biota before and after feeding. The increased richness in *Culex*, *Armigeres*, and *Toxorhynchites* challenges the paradigm that the harsh, oxidative environment of the midgut—typically dominated by *Enterobacteriaceae* and *Flavobacteriaceae*—necessarily reduces overall species count [43]. Our data, however, indicate a higher species richness following blood feeding and egg laying, even as the Shannon diversity index reflects a lower overall evenness. This suggests a dual effect: the high-nutrient environment post-feeding may allow a greater variety of bacteria to persist or temporarily colonize the gut (increasing richness), while concurrently fostering the substantial proliferation and dominance of a core group of primary symbionts that are well-adapted to the new conditions (decreasing evenness). The use of aseptic sheep blood via artificial feeders may have minimized environmental microbial noise, providing a clearer view of these internal shifts. While pathogens have evolved to manipulate the vector gut microbiota [51], the microbiota has counter-evolved to prevent their establishment [52]. Although we have mapped these compositions and their haemolytic potential, their full functional dynamics require further investigation.

Conclusion

This research provides a comprehensive comparative assessment of the gut microbiota in vector and non-vector mosquitoes, revealing how feeding status dictates microbial community architecture. We demonstrated that while the blood meal serves as a primary driver of taxonomic shifts, the response of haemolytic bacteria is species-specific. Furthermore, the significant presence of haemolytic bacteria in the non-haematophagous *Tx. splendens* highlights their functional versatility in processing non-blood-

based nutrients. Our study supported a model of selective dominance, where nutrient-rich environments foster the proliferation of specialized core symbionts at the expense of community evenness. These insights into the taxonomic and haemolytic landscapes of the mosquito midgut provide a critical foundation for understanding the bottlenecks governing pathogen transmission. Future research employing meta-transcriptomics and metabolomics is required to translate these structural shifts into a functional map of host-microbe interactions, ultimately giving inputs for the development of microbiome-based vector control strategies.

Declarations

Ethics approval and consent to participate

This study has been excepted from ethical approval.

Consent for publication:

Not applicable.

Competing interests:

Not applicable.

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Author Contribution

MM designed the study and written the manuscript. VA, MA and SN conducted the laboratory works. BG and NK done data analysis. RP reviewed the manuscript. All authors read and approved the final manuscript.

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Data Availability

The raw data of metagenomic 16S rRNA datasets generated in this study have been deposited in the Sequence Read Archive under accession number BioProject …….. (in process). The rRNA sequence data of cultured bacterial isolates are deposited at NCBI gene bank as cited in the table 1. The datasets generated during/or analzed during the current study are available from the corresponding author upon request.

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Figures

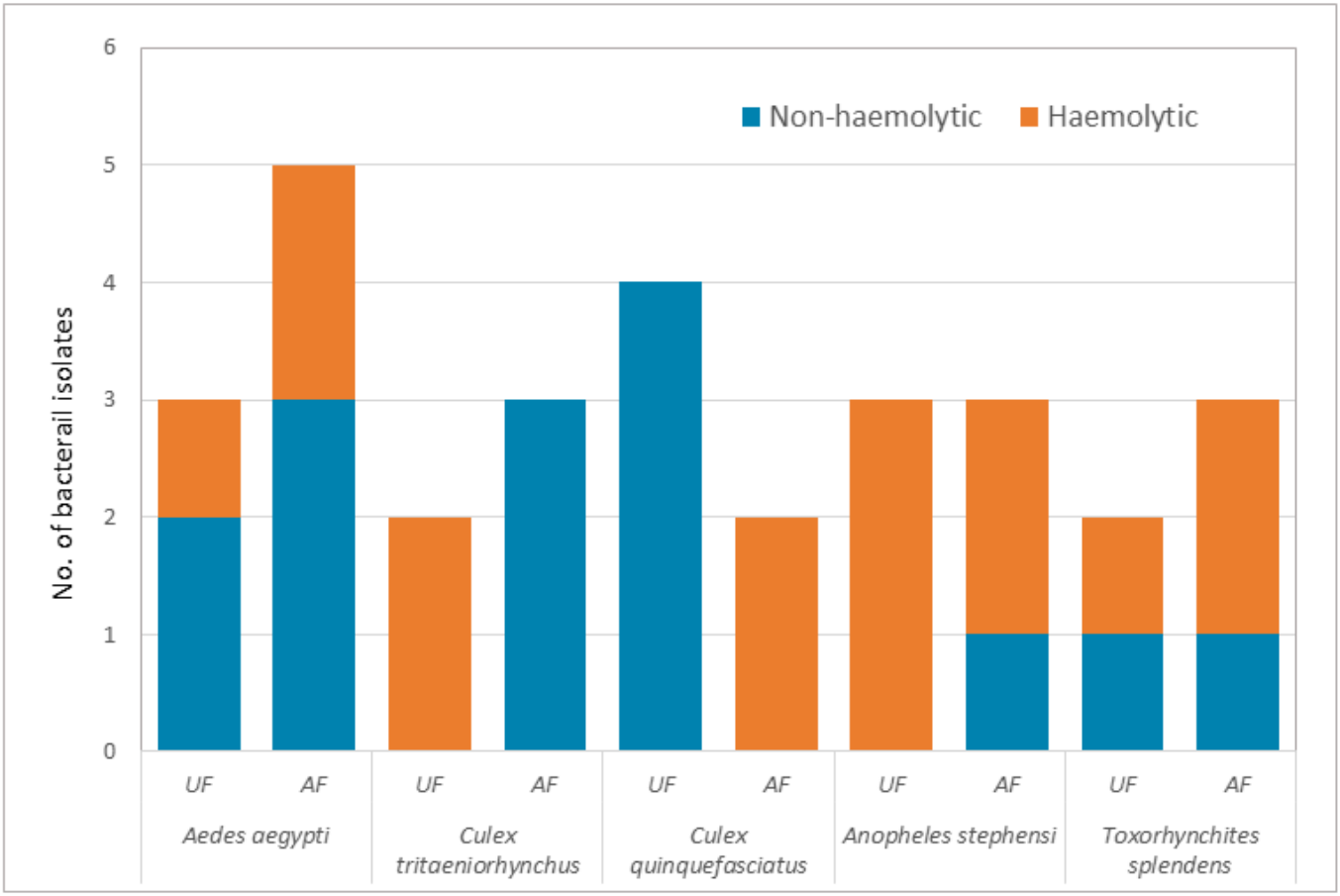


Figure 1

The distribution of haemolytic and non-haemolytic bacterial communities in different mosquito species.

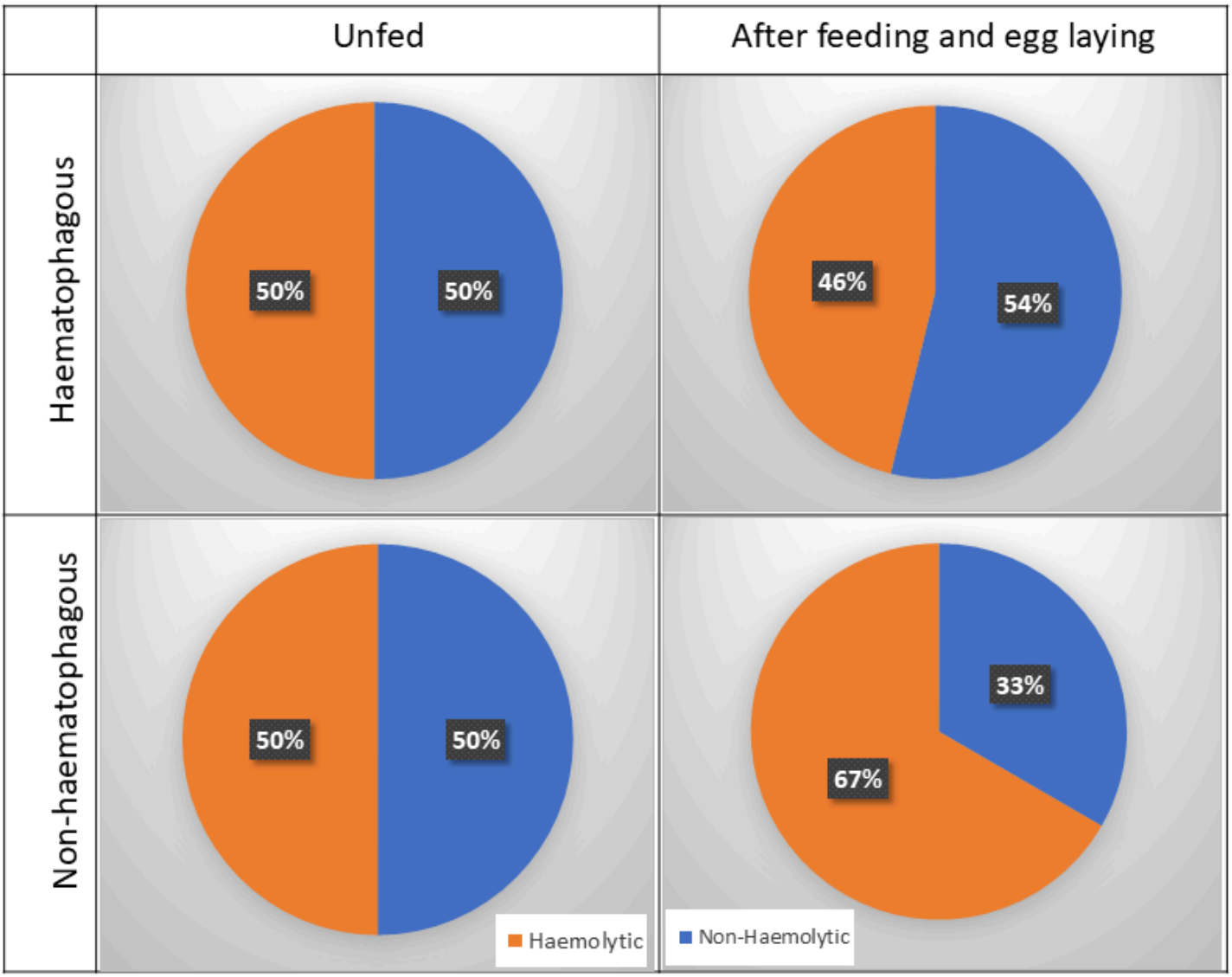


Figure 2

Changing distribution of haemolytic and non-haemolytic bacterial communities in the haematophagous and non-haematophagous mosquitoes after feeding.

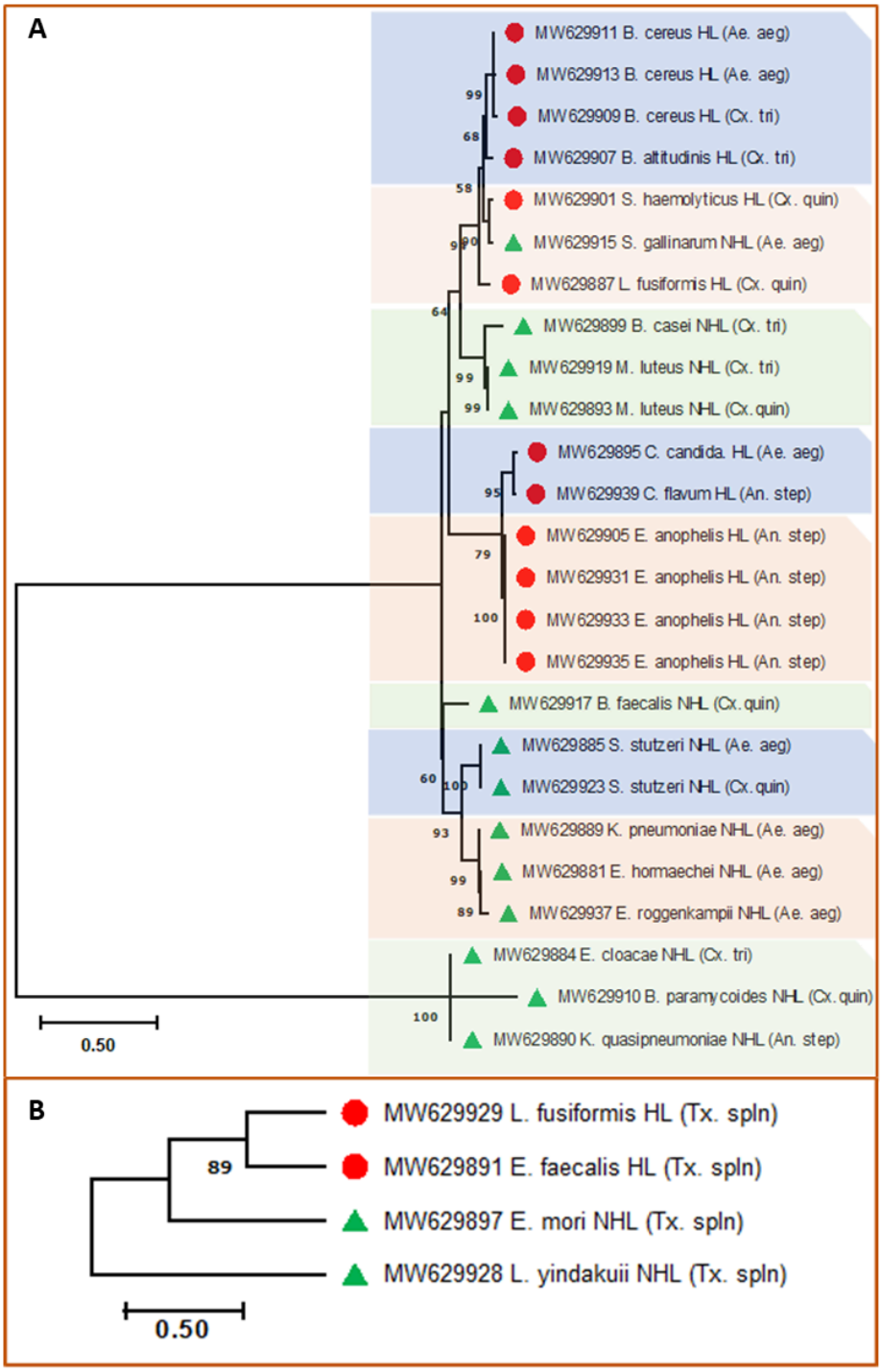


Figure 3

Maximum likelihood (ML) phylogenetic analysis of haemolytic and non-haemolytic bacteria associated with hematophagous mosquitoes (A) and Neighbour Joining (NJ) phylogenetic analysis of haemolytic and non-haemolytic bacteria associated with Non-hematophagous *Toxorhynchites splendens* mosquito (B). The phylogenetic tree was constructed in MEGA 11 using the Kimura 2-Parameter model (K2P) with gamma distribution sites (K2P+G). Bootstrap support values were calculated from 500 replicates and

are shown at the corresponding nodes. Haemolytic bacterial isolates are indicated by red circles, while non-haemolytic isolates are represented by green triangles.

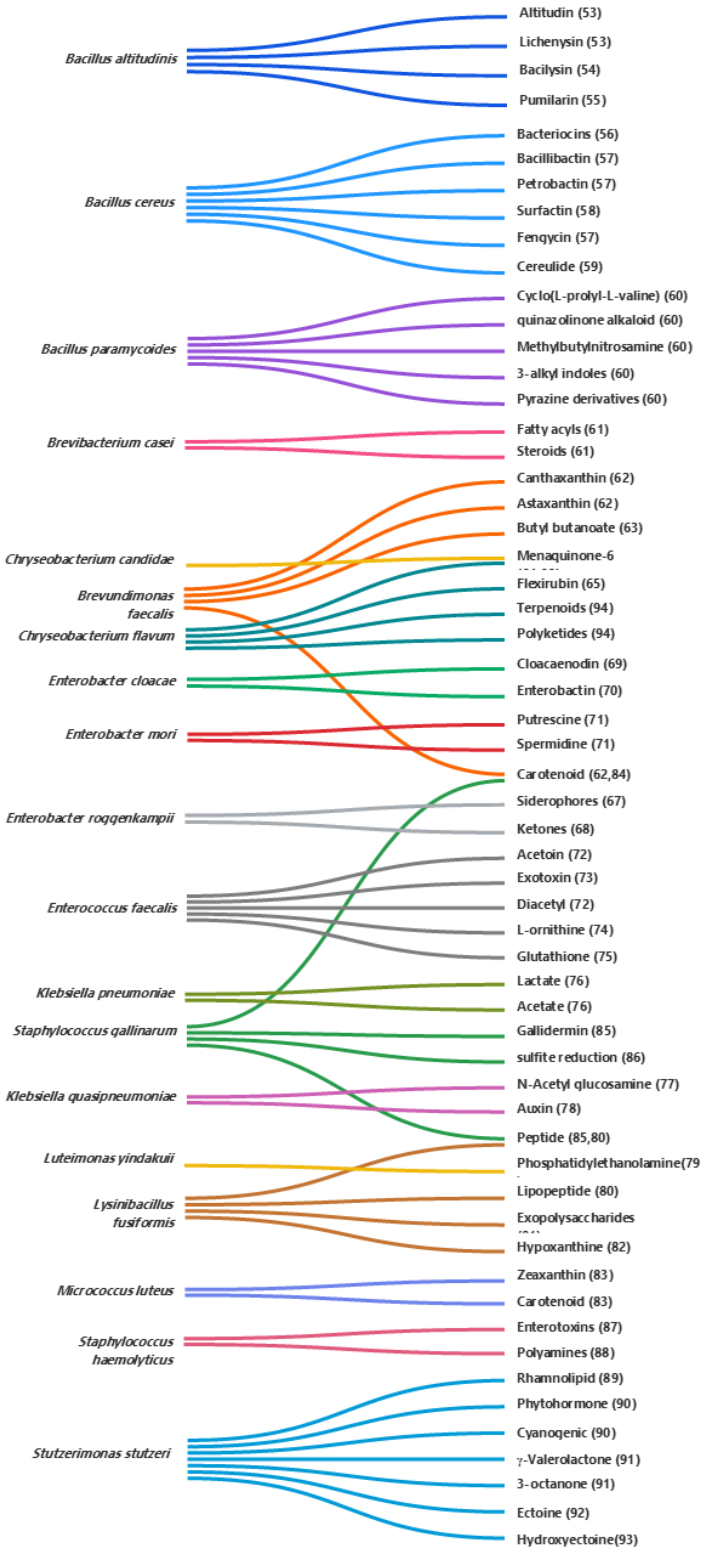


Figure 4

Metabolic compounds released by the mosquito gut bacterial isolates. Reference is cited in parenthesis.

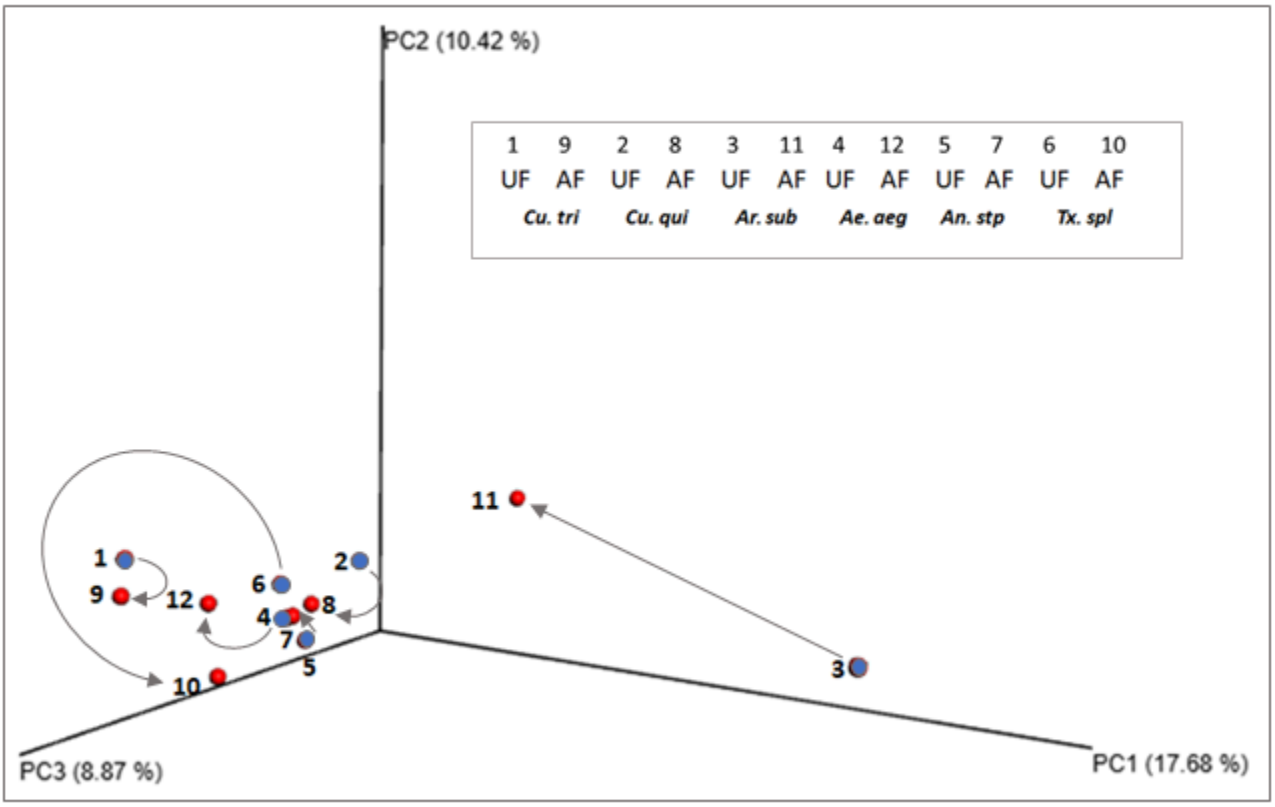


Figure 5

Unweighted unifrac PCoA plot showing the changing dynamics in the richness of the gut bacterial community after feeding. Blue dots -unfed, Red dots -after feeding and egg laying state of mosquitoes; the arrows indicate the position of gut bacterial community after feeding. The inset shows sample numbers (1-12) with feeding status (UF: unfed; AF: after feeding and egg laying) and corresponding mosquito species.

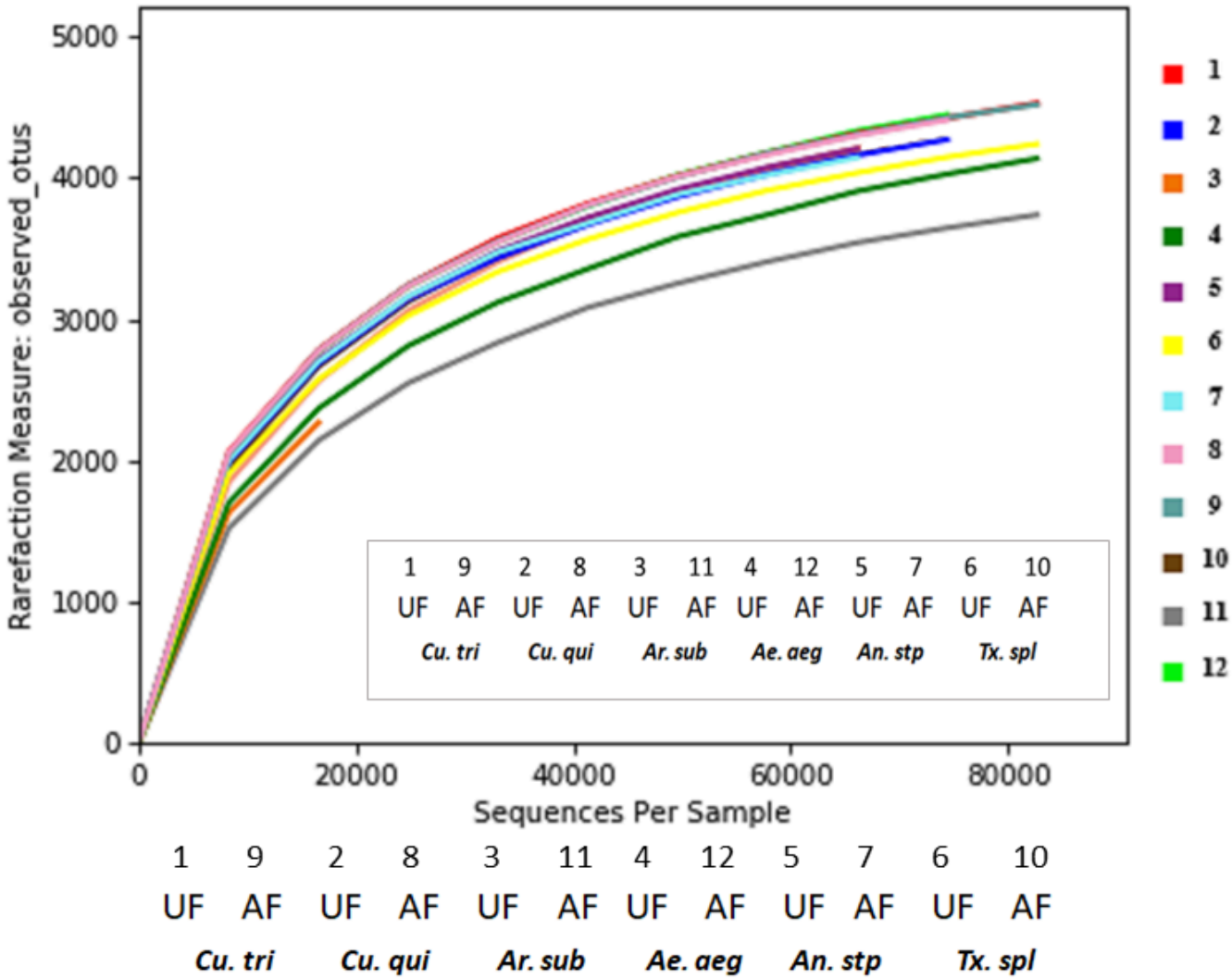


Figure 6

Rarefaction analysis showing a steep initial rise of curves followed by a gradual approach towards saturation. It shows that the sequencing depth was sufficient to capture the majority of the bacterial diversity in each sample. The inset shows sample numbers (1-12) with feeding status (UF: unfed; AF: after feeding and egg laying) and corresponding mosquito species.

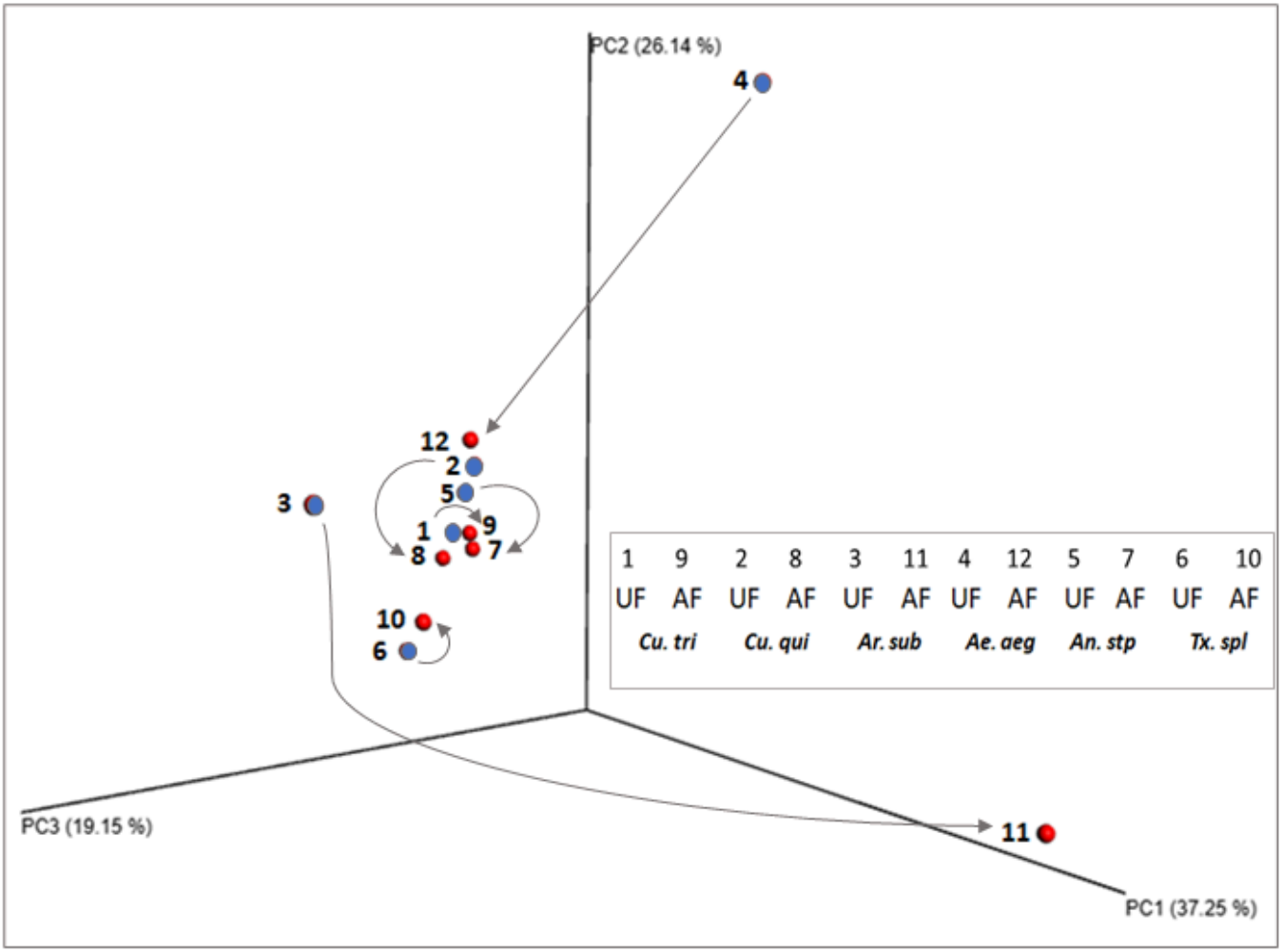


Figure 7

Weighted unifrac PCoA plot showing the relative abundance for gut bacterial communities in mosquito species after feeding. Blue dots –unfed, Red dots - after feeding and egg laying state of the mosquito species. The arrows indicate the position of the gut bacterial community after feeding. The inset shows sample numbers (1-12) with feeding status (UF: unfed; AF: after feeding and egg laying) and corresponding mosquito species.

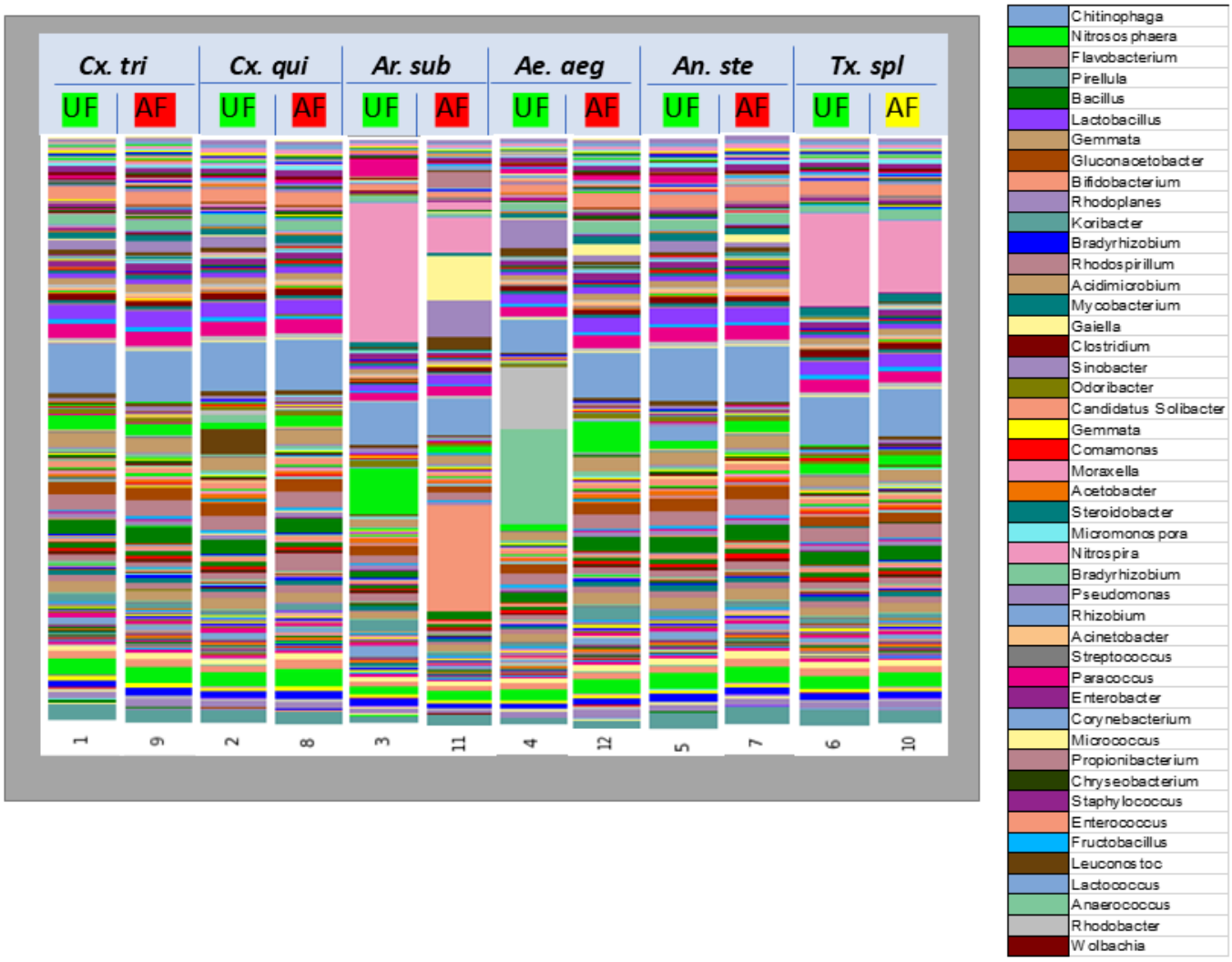


Figure 8

Relative abundance of gut bacterial communities in mosquitoes, *Culex tritaeniorhynchus* (Cx. tri), *Culex quinquefasciatus* (Cx. qui), *Armigeres subalbatus* (Ar. sub), *Aedes aegypti* (Ae. aeg), *Anopheles stephensi* (An. ste), *Toxorhynchites splendens* (Tx. Spl) during the unfed stage (UF) and after feeding and egg laying (AF) at the genus level.

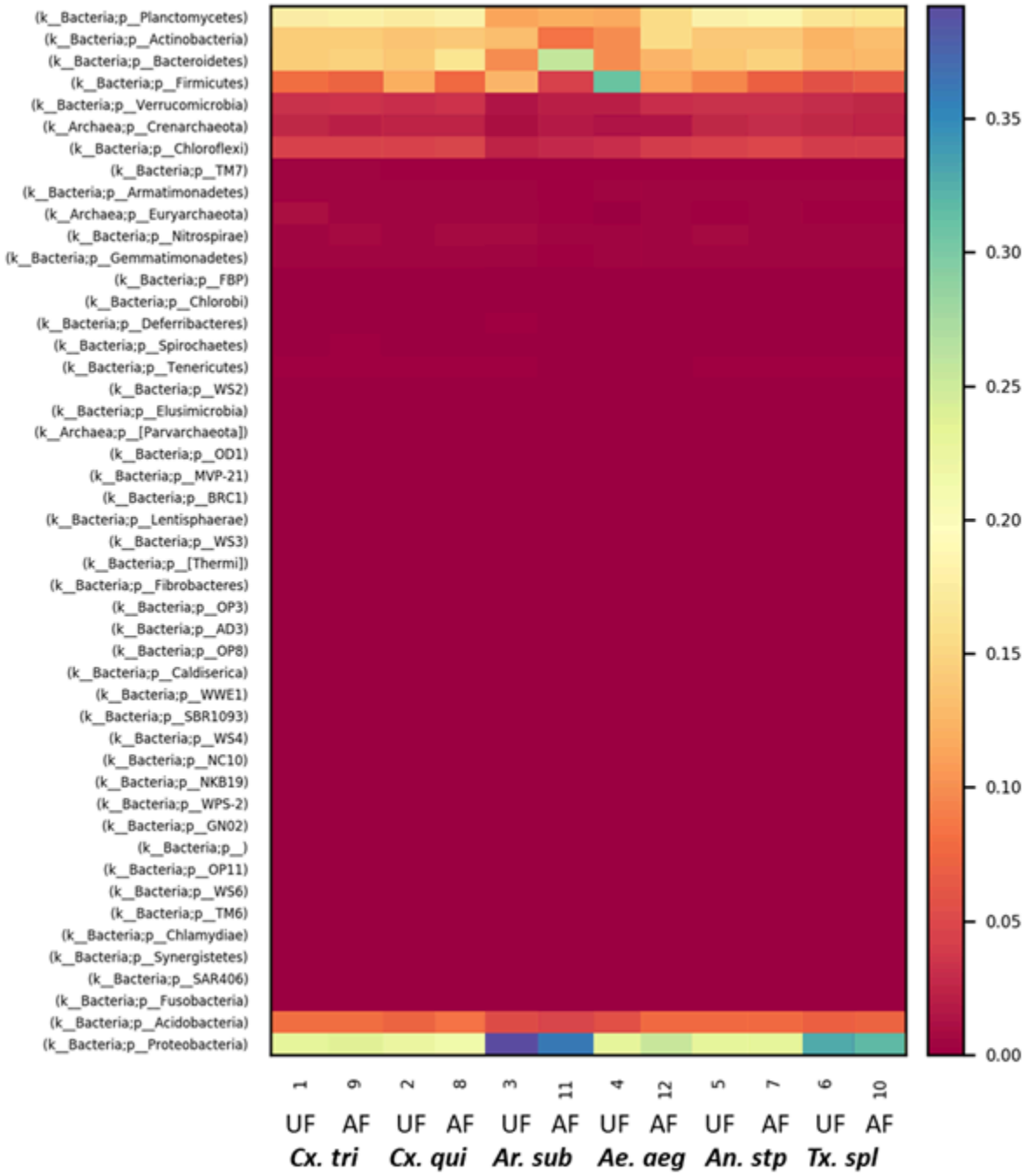


Figure 9

The relative abundance of different bacterial phyla in the gut microbiome community of mosquito species during the unfed (UF) and after feeding (AF) state. Values are scaled by the relative abundance of the bacterial phyla across all samples.

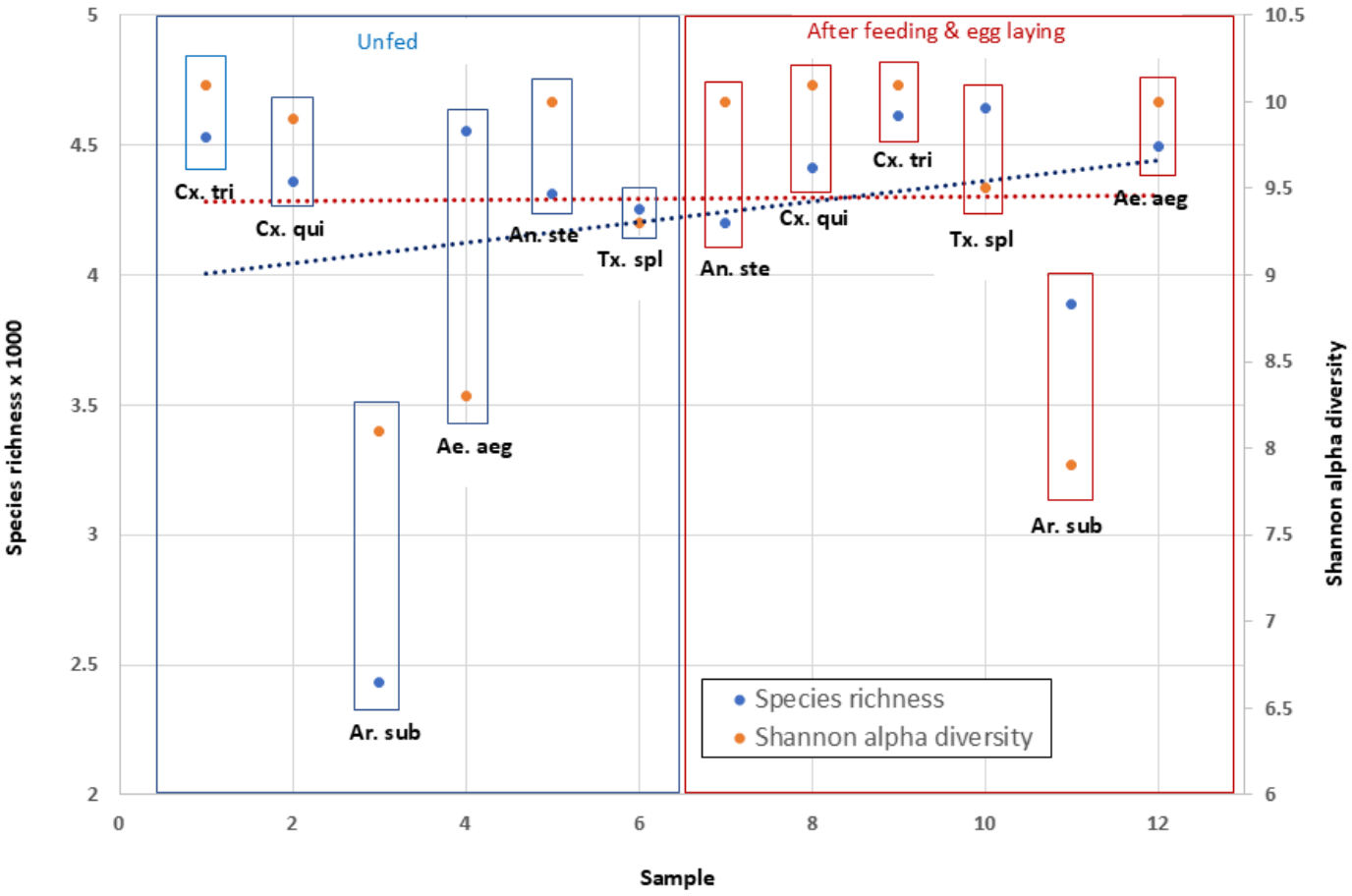


Figure 10

Changes in the relative abundance and species richness of the gut bacterial community of mosquitoes before and after feeding. The vertical square connects the blue (species richness) and orange (Shannon alpha diversity) dots of a mosquito species. The trendlines show the trend of species richness and Shannon alpha diversity of the gut bacterial community before and after feeding.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table12.pptx