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Characterization of midgut microbiome of *Anopheles stephensi* Liston

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ABSTRACT

Background & objectives: *Anopheles stephensi* is an important vector of malaria in South East Asia. The abundance and diversity of gut microbiota in the disease vectors affect their development, digestion, metabolism and immunity. The immatures of *An. stephensi* engulf microbes from their aquatic environment. The present study investigates midgut microbiota of wild and laboratory populations and compares it with their habitat bacterial diversity to study transstadial transmissibility.

Methods: The gut microbes from immatures, adults and water samples were cultured at ambient conditions on different media. The colony and biochemical characteristics, and 16S rRNA gene sequencing of gut microbes were studied.

Results: Altogether, 298 bacterial isolates were characterized as 21 genera belonging to four major Phyla viz., *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. In the field population-1, *Proteobacteria* and *Firmicutes* accounted for 49% and *Actinobacteria* constituted 51% of the bacterial isolates. In field population-2, *Bacteroidetes* and *Firmicutes* accounted for 99% of the isolates. In the laboratory populations, *Firmicutes* constituted 77%, while *Proteobacteria* 23% of the isolates. Additionally, 9 genera occurred in the breeding habitats, 13 in the larval midgut, 6 in pupal midgut, 9 in male midgut and 10 in the female midgut.

Interpretation & conclusion: This is a unique study on diversity of microbiota of *An. stephensi* from breeding water, developmental stages and adults. Different culture media used enhanced the isolation of diverse bacteria. The presence of *Micrococcus* and *Leucobacter* in different life stages indicates their adaptation in *An. stephensi* as symbionts which need further evaluation for their role in paratransgenesis.

Key words Mosquito; microbiota; diversity; midgut; transstadial

INTRODUCTION

Around 85% of malaria cases were reported in nineteen countries, i.e. in India and 18 African countries in 2018. Globally, 53% of *Plasmodium vivax* burden was in South East Asia region with 47% of cases reported in India[1]. *Anopheles stephensi* pose a major threat to human health as malaria vector, in urban areas globally and in several small, medium and metropolitan cities of India[2]. Some 0.84 million malaria cases and 194 deaths were reported by the National Vector Borne Disease Control Programme in 2017, with the highest number of cases reported from Odisha state[3].

An. stephensi prefers to breed in manmade habitats viz., wells, fountains, masonary tanks, overhead tanks, groundwater tanks, tyres, barrels, tins, intra domestic containers and curing water in construction sites[2, 4–5]. A previous study has showed that 0.1 to 0.4 percent of all curing waters stagnant in the construction sites in Goa, India acted as breeding habitats for *An. stephensi*[6]. Breeding sites are rich source of microflora and their microbial content varies depending on the depth and type of water bodies and other abiotic factors.There is a probability of intake of these microorganisms by immatures from the breeding habitats during filter feeding and subsequently becoming a part of their gut micro flora[7].

Earlier investigations had demonstrated diverse influences of microbiota on the vector competence of mosquitoes. For instance, in *Anopheles gambiae,* removal of the gut microbiome increased its susceptibility to *Plasmodium falciparum* infection. On the contrary, Boissiere *et al*. demonstrate a positive correlation between the abundance of *Enterobacteriaceae* family in the midgut of mosquito and the *Plasmodium* infection status[8–9]. Microorganisms like *Bacillus* and *Staphylococcus* help in maintaining the normal and high fecundity in *Culex pipiens*[10]. Similarly, *Wolbachia* is also known to play an important role in enhancing the rate of vertical transmission in the host by manipulating the Cytoplasmic incompatibility[11]. Also, microbiota has been found to play a significant role in determining the vectorial capacity of the Anophelines[8–9, 12].

The composition of the midgut microbiome may vary due to diverse factors and one of them which can directly influence the vector microflora diversity is the breeding

habitat. Hence, understanding the correlation between the type of breeding habitat and its influence on midgut microbiome remains an exciting area of research. Paratransgenesis is an upcoming vector control strategy that uses mosquito associated microbiome for the control of vector borne diseases. Screening of isolates for paratransgenesis is routinely carried out using culture methods. For example, the introduction of *Asaia,* which induces the activation of mosquito immunity significantly, reduces the development of malaria parasite in *An. stephensi*[13]. This strategy could be exploited to combat malaria[14].

The present study focuses on the isolation and characterization of microflora using the cell culture techniques as has been previously attempted successfully[8, 15–18]. Among all the techniques, routine culture method provides deeper insight into the different characteristics of the bacteria. Hence, this study will not only elucidate the influence of breeding habitats on vector competence, but will also provide information on whether there is a transstadial transmission of the gut microbiota across various stages of the mosquito life cycle. The deciphering of the gut microbiota would be a first preliminary step towards its manipulation as a tool in the integrated vector management and the control of vector borne diseases.

MATERIAL & METHODS

Study area and time

The wild larval populations were sampled from a construction site located in Ponda city of Goa, India. The larval collections were made from the puddle (FS-1) and Curing waters on cement slabs (FS-2). The sampling of the larvae was carried out from FS-1 in pre-monsoon and from FS-2 in post-monsoon period during May 2015 to August 2016. For a comparison, the laboratory reared larval population was obtained from the cyclic colony of *An. stephensi* maintained at National Institute of Malaria Research, Field Unit at Goa.

Collection and maintenance of An. stephensi immatures from the breeding habitats

The larvae of *An. stephensi* were separated and identified based on their morphology and distinct mobility pattern.The identity of the species was further confirmed taxonomically from adults reared from the pupae obtained from the sampling sites. The immatures were collected from the breeding sites in sterile containers using the dipping technique[19]. Similarly, water samples from the breeding site were also collected for the maintenance of immatures in the laboratory. The collected specimens along with breeding water were labelled, transported and reared in the insectary of National Institute of Malaria Research, field unit at Goa, India.

Maintenance of An. stephensi immatures in the laboratory conditions

The third and the fourth instar larvae were separated from the population collected from the breeding sites and reared in separate sterile bowls that were suitably labelled. The rearing of immatures was done in the field collected water brought from their respective breeding habitat (FS-1 & FS-2).

Pupae were collected from the breeding sites in a 500 ml plastic bowls containing 200 ml sterilised water and were placed inside a closed cage measuring 10 cubic feet for the emergence and identification. The immatures were reared in the plastic trays under laboratory conditions at 27±2°C, 70 ± 5% relative humidity and 12h light and 12h dark photo period cycle. A pinch of sterilised food, a combination of Cerelac™ powder (Nestle) and fish food in a ratio of 1:1, was given to the larvae once a day until pupal stage. On emergence, the species of adult mosquitoes were identified using standard taxonomical keys[20]. Freshly emerged adults were offered 10% sterilised glucose solution soaked in a sterilised cotton pad for the first 24 hours.

Surface sterilisation and midgut isolation from immatures and adults

For the isolation of microbiota, the larvae, pupae, adults (male and female) were surface sterilized by immersing into Sodium hypochlorite solution (10%) for a minute followed by immersion into alcohol (70%) for five minutes. They were washed with Phosphate Buffer Saline (PBS) four times to remove all traces of alcohol. The sterile immatures and adults were placed on a sterile slide containing a drop of normal saline for dissection. The dissected midguts isolated from the larvae, pupae, adults (male and female) of *An. stephensi* from different sites (lab and the two field samples) were then collected in the sterile Eppendorf tubes containing PBS (using modified protocol)[15, 19]. The numbers of midguts dissected from each stage are given as supplementary information S1.

Isolation of microbiota from the midgut of immatures and adults of An. stephensi

The dissected midguts were completely homogenised with the sterile pestle and Eppendorf tubes in PBS and the extracts were serially diluted up to 10^{-5} dilution. Each dilution was spread onto five different media *i.e.* Blood Agar (BA), Brain Heart Agar (BHA), Nutrient Agar (NA), Luria Berthani Agar (LBA) and Yeast Extract Agar (YEA)

plates[9, 15, 19, 21].The breeding water sample was also diluted and spread onto these media. The culture plates were incubated for 24–48 hr at 30°C. After incubation Total Viable Count (TVC) was recorded and the isolates were picked up based on their differences in colony morphology. The isolates were re-streaked onto respective agar medium to obtain the pure cultures. The purified colonies were maintained at 4°C.

Morphological and biochemical characterization of the isolates

The isolates were screened based on their colony characteristics including size, shape, colour, elevation and texture. The cell morphology of the isolate was determined using Gram staining. Microbial isolates were further characterised based on biochemical tests namely Glucose utilization, Citrate, utilisation, Catalase, Methyl Red, Vogues Proskauer, Nitrate Reductase and Gelatinase production[15].These tests were conducted for the preliminary identification of the isolates following the Bergey's Manual. Initial grouping and clustering of the isolates were based on their colony morphology, gram characteristics and biochemical methods[15–16, 19].

Extraction of genomic DNA and 16S rRNA gene amplification of the isolates for molecular identification

The 298 purified bacterial isolates were grown for 24 hr, in respective media at 30°C. DNA was extracted using the protocol as described[22]. Gene sequences of 16S rRNA was amplified using the universal primers, (Forward: S-D-Bact-0011-a-5-17:5`-GTTTGATCCTGGCTCAG-3 & Reverse: S-*-Univ- 1392-b-A-15: 5`-ACGGGCGGTGTGTNC-3`). Amplification of 1.5 kilo base (kb) of the 16S rRNA gene was carried out from all of the DNA samples extracted from individual bacterial isolates. The PCR conditions were set as an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 mins, followed by a final extension at 72°C for 5 min. All successfully amplified 16S rRNA amplicons were purified using QIA quick PCR purification kit and QIAquick Gel extraction kit. The 16s rRNA were then sequenced using illumina Miseq platform.

Sequencing and phylogenetic analysis

In order to identify the bacterial species, sequences obtained in the study were compared with the Genbank database using the BLAST algorithm and were submitted to the GenBank under the accession numbers as given in (Supplementary information S2). Multiple sequence alignment of partial 16s rRNA gene sequence was performed and the phylogenetic relatedness of 298 sequences were analysed by using Molecular Evolutionary Genetic Analysis (MEGA 10.0.05). Phylogenetic analysis was performed using Maximum Likelihood method and Tamura-Nei model, with a bootstrap analysis of 1000 replicates. *Sulpholobus solfataricus* (Accession no. X03235) was selected as an out-group[15].

Statistical analysis

In order to account for the number of genera present in the different stages of the midgut and breeding water, the diversity indices like Shannon diversity, Simpson's diversity and Shannon Evenness was determined. These indices assist in assessing bacterial generic diversity obtained from the culture based method. Species diversity and evenness were calculated for all the isolates at different developmental stages. Simpson's Diversity was calculated using the formula given by Mohlmann[23].The presence of each isolate on the same plate was considered for calculation of the indices (Supplementary information S2). Simpson's Diversity reflects the probability that two individuals taken randomly from the dataset are not the same species. Values for Simpson's diversity range between 0 and 1, with larger values representing greater diversity. The Shannon Diversity is based on the uncertainty that an individual taken at random from the dataset is predicted correctly as a certain species. Larger values represented larger uncertainty, thus greater diversity. Shannon evenness was also calculated, the values ranged between 0 and 1; where 1 is complete evenness, i.e. all species being equally abundant[23].

RESULTS

Microbial diversity and abundance was calculated for different stages of *Anopheles stephensi* along with breeding water of both laboratory reared and wild population. Overall a total of 340 midguts were dissected from the larvae, pupae, male and female of both laboratory reared and wild population of *An. stephensi*. Total no. of colonies isolated on each medium plate for different stages were used to determine the total viable count (TVC). A total number of 4744 isolated colonies were obtained from all the stages and different sites, which were then characterised using biochemical and molecular tools.

Total viable count (bacterial abundance)

The midgut homogenate was spread plate onto five different media, the TVC was calculated for each stage on all five media. The highest bacterial count of 2.40 x 10^5cfu/midgut was obtained from the larval midgut of

FS-2 sample on BA. The least bacterial count of 2.38 x10^{2}cfu/midgut was obtained from the midgut of laboratory reared larvae on YEA media. The midguts isolated from the male of laboratory population of *An. stephensi* showed, the highest bacterial count followed by female, pupae and larvae on LBA, BHA and YEA media respectively as shown in (Fig. 1). In case of NA and BA the highest count was seen in the male midgut followed by female, larvae and pupae.

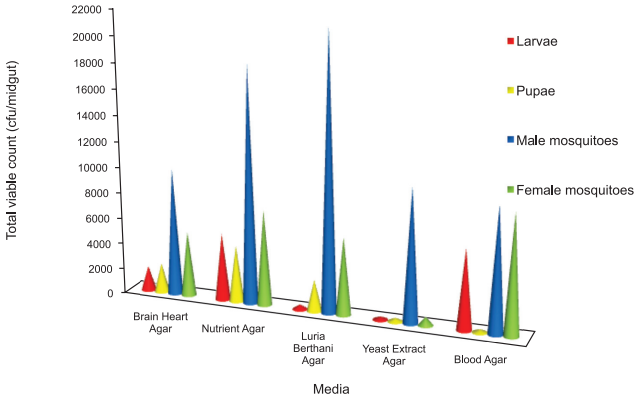

Fig. 1: Comparative Total Viable Count of midgut of different life stages of *Anopheles stephensi* in the laboratory population on five different media

In case of water collection site (FS-1 sample) population, the highest bacterial count of 15 x 10^{5}cfu/midgut was obtained from the pupal midguts on BA as shown in (Fig. 2). The least count of 2.75 x 10^{2}cfu/midgut was obtained from the midguts of male on BA. In case of LBA and YEA, the larvae showed the highest count followed by the pupae. On LBA medium, both male and female midguts showed the similar bacterial count. Bacterial count on YEA was higher in male than in female. On NA and BA media, the pupae midgut showed highest TVC followed by larvae with male and female adults showed similar counts. Bacterial count obtained from the female midgut was greater than in male on BA. On BHA medium, the female midgut showed the highest TVC followed by pupae, larvae and male.

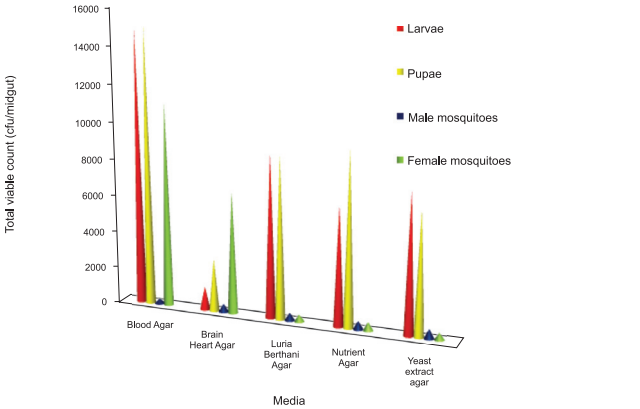

Fig. 2: Comparative Total Viable Count of midgut of different life stages of *Anopheles stephensi* in FS-1 sample population on five different media

In case of second breeding site (FS-2: curing water), a highest count of 2.4 x 10^{5}cfu/midgut was obtained from the midgut of larvae on the BA medium and the least was in the case of male midgut on YEA medium (7.82 x 10^{2}cfu/midgut) as shown in (Fig. 3). In the case of BA, LBA and NA media, the highest bacterial count was obtained from the larval midgut followed by female, pupae and male midgut. In BHA, the highest count was found in the larvae midgut followed by male, female and pupae. On YEA medium, the highest count was found in the larvae midgut followed by midgut of pupae, female and male. Different media used showed diverse viability counts, suggesting the significance of using multiple media in isolating the microflora from the different stages of the vector species and the breeding sites.

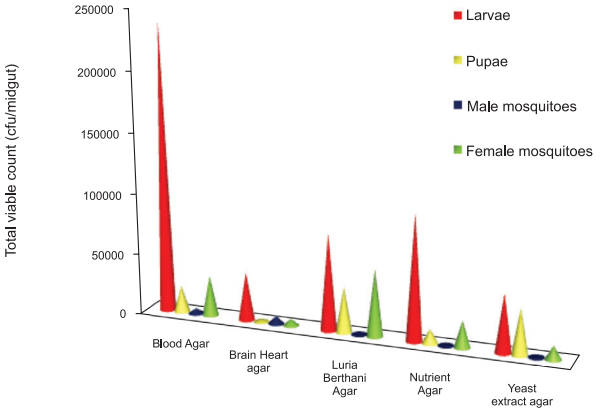

Fig. 3: Comparative Total Viable Count of midgut of different life stages of *Anopheles stephensi* from FS-2 population on five different media

Determination of midgut microbiome associated with different life cycle stages

Total no. of colonies isolated on each medium plate from different stages were used to determine the Total viable count. A total of 4744 isolates were obtained from all the stages among different sites. Altogether 298 isolates-from 4744 were selected for characterization based on biochemical and 16S rRNA sequencing method. The characterization of gut flora in *An. stephensi* from three different populations using culture dependent method led to the identification of 21 bacterial genera. These were isolated from different media and mainly belonged to four major phyla of bacteria, namely *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria*. The detailed information of every genus at each developmental stage and their accession numbers are given in the phylogenetic tree shown in (Fig. S3–S5). In FS-1 sample, Phyla *Proteobacteria* and *Firmicutes* accounted for 49% of the species, while

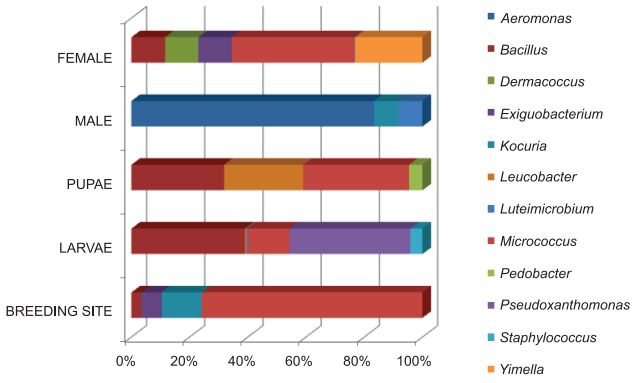

Fig. 4: Relative abundance of the bacterial genera within mosquito midguts of larval, pupal, adult stages and from the water sample of breeding site (FS-1).

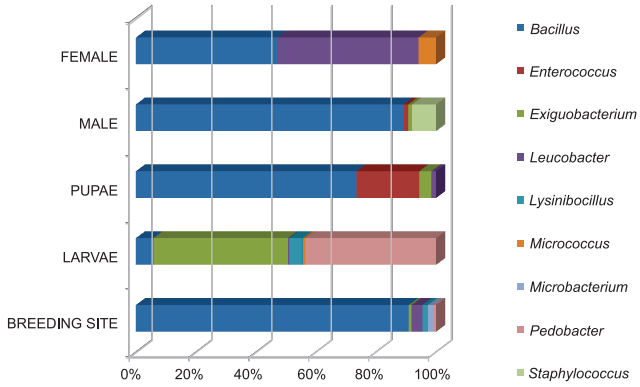

Fig. 5: Relative abundance of the bacterial genera within mosquito midguts of larval, pupal, adult stages and from the curing water breeding habitat sample (FS-2).

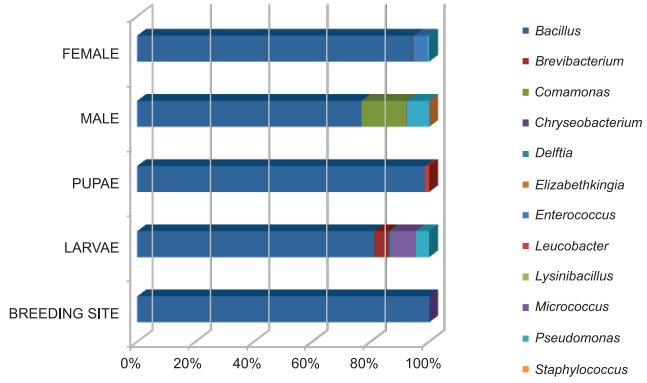

Fig. 6: Relative abundance of the bacterial genera in midguts of larval, pupal, adult stages and water used for rearing *An. stephensi* in the laboratory.

Actinobacteria constituted about 50% of the total bacterial isolates. In case of FS-2 sample, *Bacteroidetes* and *Firmicutes* accounted for 99% of the isolates. While, in the Laboratory population, *Firmicutes* constituted about 77% of total isolates with 23% of *Proteobacteria*. A total of 9 genera were observed from the breeding habitat of *An. stephensi*, 13 from the larval midgut, 6 from the pupal midgut, 9 from the male midgut and 10 from the female midgut. Overall, the most predominant Phylum was found to be *Firmicutes,* which was recorded in all the stages of mosquitoes of both laboratory and wild population.All the different isolates were clustered (grouped) in the phylogenetic tree analysis as shown in (Fig.S3).

Microbiota of breeding habitats

As for bacterial isolates from water samples, four bacterial genera were detected in FS-1 sample, six in FS-2 sample and two in the laboratory water sample as shown in (Fig. 4–6). Among all the isolates obtained from the breeding habitat, *Bacillus* was isolated from all three sites. The least count was found in FS-1 water sample, followed by FS-2 sample and Laboratory sample. *Exiguobacterium* was found in FS-1 (67%) and FS-2 (33%) samples. *Micrococcus* and *Kocuria* were present in the water sample from FS-1 site. While *Leucobacter*, *Lysinibacillus*, *Microbacterium* and *Pedobacter* were identified only from the breeding habitat water of the FS-2, with an exception of *Kocuria* which was reported in FS-1 site only.

Midgut microbiota of the larvae of An. stephensi

The larvae midgut showed presence of genera *Bacillus* and *Micrococcus* in all three sampling sites. *Bacillus* was identified with highest percentage (81%) in LAB population followed by FS-1sample (39%) and FS-2 sample (6%) respectively. *Leucobacter* growth was seen higher in FS-2 in comparison to FS-1. While *Lysinibacillus* was found more dominant in FS-2 (5%) followed by lab population.

Pseudoxanthomonas, *Staphylococcus*, and *Luteimicrobium* were exclusively found in FS-1 samples.Also, *Pedobacter* was exclusive to FS-2 larval midguts. The genera present only in the laboratory population included *Pseudomonas*, *Brevibacterium*, *Delftia* and *Chryseobacterium*.

Midgut Microbiota of the pupae of An. stephensi

The pupal midgut showed *Bacillus* and *Leucobacter* in all three samples. Besides these, *Pedobacter* (6%) and *Micrococcus* (47%) were present only in the pupal midgut of FS-1 population. *Exiguobacterium* (6%) and *Enterococcus* (32%) were recorded only in the pupal population of FS-2 sample.

Midgut microbiota of adult male An. stephensi

In case of the male midgut, *Bacillus* and *Staphylococcus* were the only two genera isolated from FS-2 and lab population. Genera reported from FS-1 male midgut included, *Aeromonas, Kocuria* and *Luteimicrobium*. In the

FS-2 sample population, *Staphylococcus*, *Enterococcus*, and *Exiguobacterium* were observed. Similarly, *Comamonas*, *Pseudomonas* and *Chryseobacterium* were found in the male midgut of the lab population of *An. stephensi*.

Midgut microbiota of adult female An. stephensi

In the female midgut, the only genus recorded from all three samples was *Bacillus*. It was predominant in lab population, followed by FS-1 and FS-2 samples. *Micrococcus* was reported from the female midgut of FS-1 and FS-2 populations. While *Exiguobacterium, Dermacoccus*, *Micrococcus*, *Staphylococcus* and *Yimella* were found in the female midgut of FS-1 sample. *Leucobacter* was the only genus reported from FS-2 sample population. The female midgut from lab population showed abundance of *Enterococcus*, *Pseudomonas* and *Elizabethkingia*.

Overall, a multitude of culturable midgut isolates were reported for the first time in *An. stephensi* and these included *Delftia, Leucobacter, Luteimicrobium, Micrococcus, Pedobacter* and *Staphylococcus*. All these were isolated from the larval midgut of *An. stephensi*. While, *Leucobacter, Micrococcus* and *Pedobacter* were isolated from the pupal midgut of *An. stephensi, Aeromonas, Comamonas*, *Exiguobacterium, Luteimicrobium, Pseudomonas* and *Kocuria* were reported from the midgut of male *An. stephensi*. In the female midgut, the bacterial isolates like *Elizabethkingia, Microbacterium* and *Staphylococcus* were reported for the first time. Similarly, *Exiguobacterium, Kocuria, Micrococcus* and *Microbacterium* were the isolates reported for the first time from the mosquito breeding habitat.

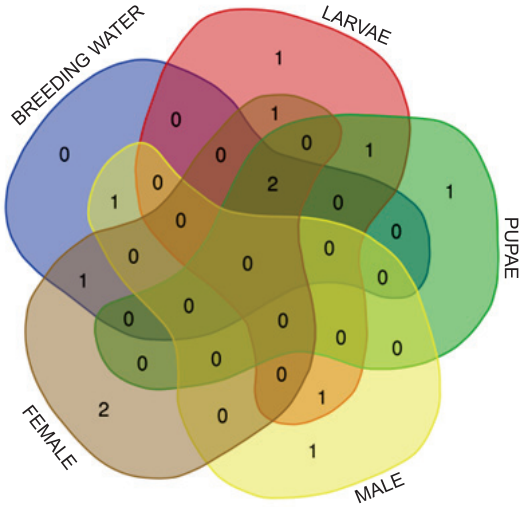

Fig. 7: Venn diagram presenting distinctive and common microbial species between the larval, pupal and adult stages of *Anopheles stephensi* isolated from the wild population sample of field site 1.

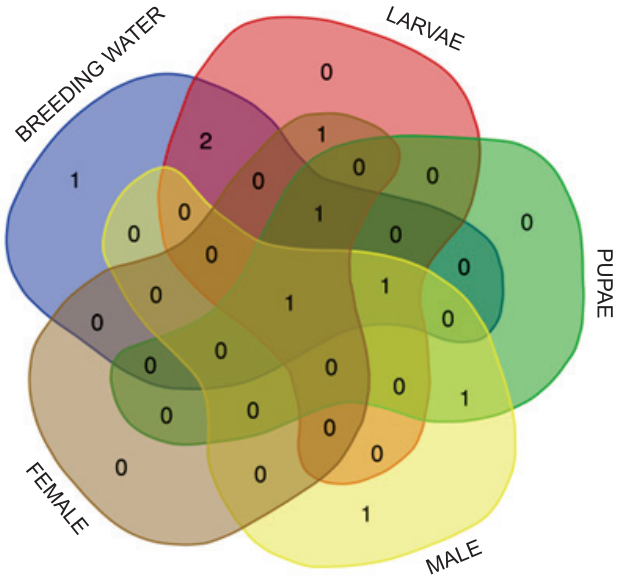

Fig. 8: Venn diagram presenting distinctive and common microbial species between the larval, pupal and adult stages of *Anopheles stephensi* isolated from the wild population sample of field site 2.

Luteimicrobium is isolated for the first time from the midgut of mosquito; previously, it was identified from the long horned beetle[24]. To the best of our knowledge, this is the first study conducted on the pupal stage of *An. stephensi,* earlier study on pupae in Anophelines were conducted on *An. gambiae*[17,25].

Microbiota common to breeding habitat and life stages of An. stephensi in FS-1 population

Some isolates were seen only in one stage, while some were recorded in more than two developmental stages. As seen (Fig. 7), there were only two isolates common in breeding water, larvae, pupae and female of wild *An. stephensi* population (FS-1) stage viz., *Bacillus* and *Micrococcus*. *Kocuria* was found in both breeding habitat water and in the midgut of male. *Exiguobacterium* was obtained from the breeding habitat water and the midgut of the female. *Leucobacter* was a common isolate in the midgut of larvae and pupae. *Luteimicrobium* was found common in the midgut of the adult male and larvae. *Staphylococcus* was common isolate in the midguts of larvae and adult female.The isolate specifically found in larvae, pupae and male midgut was *Pseudoxanthomonas, Pedobacter*and *Aeromonas.Dermacoccus* and *Yimella* were isolated from the female midgut of FS-1 sample population.

Microbiota common to breeding water and life stages of An. stephensi in FS-2 sample population

Table 1. Diversity indices of midgut bacterial isolates of different life stages of *An. stephensi* and the breeding habitat

Source of Microbiome	Sampling Site	Simpsons Diversity	Shannon Diversity	Shannon Evenness	Total no. of Genera
Breeding Water	FS-1	0.365	0.736	0.531	4
	FS-2	0.109	0.306	0.171	6
	LAB	0.006	0.022	0.031	2
Larvae Midgut	FS-1	0.642	1.193	0.666	6
	FS-2	0.595	1.064	0.594	6
	LAB	0.340	0.731	0.376	7
Pupae Midgut	FS-1	0.637	1.143	0.824	4
	FS-2	0.518	0.867	0.625	4
	LAB	0.111	0.224	0.323	2
Male Midgut	FS-1	0.292	0.566	0.515	3
	FS-2	0.204	0.432	0.312	4
	LAB	0.614	1.030	0.640	5
Female Midgut	FS-1	0.751	1.505	0.840	6
	FS-2	0.594	0.965	0.878	3
	LAB	0.260	0.480	0.347	4

In case of FS-2, *Bacillus* was reported from all three life cycle stages and also in the breeding habitats as seen in (Fig. 8). *Exiguobacterium* was reported in the breeding water, larvae, pupae and the male midgut. Similarly, *leucobacter* genus was reported in the breeding water, larvae, pupae and the female midgut. *Pedobacter* and *lysinibacillus* are two genera common in breeding water and the larvae. *Micrococcus* was seen in both larvae and female midgut and *Enterococcus* was common isolate reported in male and pupae midgut. Both *Microbacterium* and *Staphylococcus* were individually found in the breeding water and the male midgut respectively.

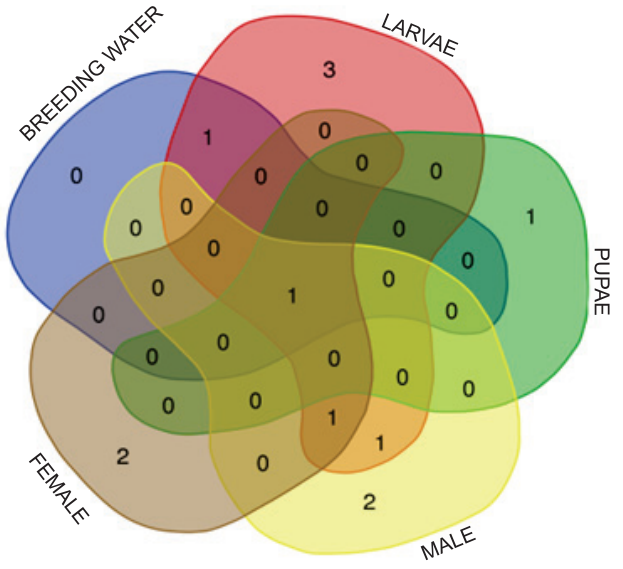

Fig. 9: Venn diagram presenting distinctive and common microbial species between the larval, pupal and adult stages of *Anopheles stephensi* isolated from the Laboratory population sample.

Microbiota common to breeding water and life stages of An. stephensi in lab population

In case of lab sample, *Bacillus* was reported from the breeding water, larvae, pupae, male and the female midgut (Fig. 9). *Pseudomonas* was reported from the larvae, male and female population. *Micrococcus* was observed in the larvae and the breeding water. *Chryseobacterium* was found common to larvae and the male midgut. *Brevibacterium, Lysinibacillus* and *Delftia* are the genera obtained from the larvae midgut while, *Leucobacter* is the only isolate obtained from the pupal midgut. *Comamonas* and *Staphylococcus* were obtained from the male midgut while *Elizabethkingia* and *Enterococcus* were reported from the female midgut. *Bacillus*, *Exiguobacterium, Micrococcus* and *Leucobacter* were found in different developmental stages as well as in the breeding water and hence could be considered as potential candidates for further investigations on transstadial transmission.

Besides the characterization of the microbiota, estimation of their abundance remains very important. The Simpson diversity, Shannon diversity and Shannon Evenness were estimated from all three sites for each developmental stage and the breeding water as summarised in (Table 1).

Diversity indices of midgut bacterial isolates of different life stages and the breeding habitat

In the present study, the value of Simpson diversity index ranged from 0 to 0.36 among breeding habitat, 0.34 to 0.64 in larvae midgut, 0.11 to 0.63 in the pupae midgut, 0.29 to 0.45 in the male midgut and 0.26 to 0.75 in the female midgut. As observed, breeding habitat may affect the diversity of microbiota in pupae and female midguts, while larval and adult male stages have resulted in a less or more consistent observed diversity suggesting stage-specific preference of microbial communities.

With regards to site variables (FS-1, FS-2 and LAB), generic diversity was generally higher in most stages of the mosquitoes collected from FS-1 site (outdoors) and generally lower in stages of mosquitoes reared on tap water in the laboratory-reared populations; with the exception of adult males which showed higher generic diversity in Lab rather than FS-1 collected. The male midgut of the lab population showed five bacterial genera out of which *Bacillus, Comamonas*, *Pseudomonas* and *Chryseobacterium* showed the highest total viable count. While in the case of FS-2 population very low Total Viable Count was shown by the bacterial genera present. Thus, result-

ing in the change in Simpson's diversity. Despite higher sensitivity of Shannon diversity, calculations revealed no observable difference with respect to Simpson's diversity; hence similar trends.

With regards to the evenness of microbial communities, the value of Shannon evenness ranged from 0.03 to 0.53 in the breeding habitat. In the case of larval midgut, the value ranged from 0.37 to 0.66, 0.32 to 0.82 in the pupal midgut, 0.31 to 0.64 in the male midgut and 0.34 to 0.87 in the female midgut. In the case of breeding water, larvae and pupae the highest Shannon evenness were noticed in FS-1 and the least in LAB population. In the case of male midgut from LAB population showed more evenness and least was observed in FS-2 population. In case of female midgut, highest diversity was observed in FS-2 and the least in the LAB population.

The high fluctuation of Shannon evenness in the lab population, suggests the uneven distribution of genera, while in the case of FS-2 the genera are well distributed. For instance, though there are three genera reported in FS-2 female in comparison to four genera in the lab population. In FS-2 population all the genera present have similar total viable count as reflected in its evenness Table 1. However, in case of Lab population higher relative abundance of *Bacillus* was observed compared to other genus resulting in uneven distribution, with an exception in male population.

DISCUSSION

The elucidation of mosquito gut microbiota is a growing field of investigation because of its potential role in vector competence to transmit disease pathogens. A protective role of bacterial microbiota of *Anopheles* sp. against *Plasmodium* infections has already been demonstrated[9, 26–27]. The present study provides an overview of the midgut microbiome isolated from different life stages and breeding environment of *An. stephensi*. This is the possibly the first study where the analysis of midgut flora has been carried out in mosquito immatures and adults of *An. stephensi* obtained from breeding habitats, water collection area and curing water site,and compared with the laboratory population. In several earlier studies on Indian and Iranian *An. stephensi* microbiota of larvae and adult stages of mosquitoes, transstadial transmission of some bacterial genera such as *Acinetobacter, Bacillus, Enterobacter, Staphylococcus, Pseudomonas, Chryseobacterium* and *Serratia sp*. has been suspected[15–17, 19, 28]. In the present study, with 16S rRNA gene sequencing, a total of 298 midgut isolates were obtained from the different developmental stages and breeding habitat of the vector. The composition of isolates in this study showed that gram- positive bacteria of phylum Firmicutes and Actinobacteria dominate the midgut flora of both laboratory and field *Anopheles* mosquito species (Supporting Information S2 & S6). In contrast, the earlier culture-based studies from different geographical regions on *An. stephensi*, *An. maculipennis*, and *Anopheles gambiae,* revealed the supremacy of Gram-negative bacteria[29]. A total of 12 genera were identified from one field sample (FS-1), 9 from another (FS-2) and 11from the midguts of different life stages of the laboratory populations. The total viable count (TVC) was also calculated for the midgut homogenate of each stage on different media. In order to capture maximum diversity, five different media having different composition were utilized in the present study[15, 19, 21]. The genera *Bacillus, Kocuria, Micrococcus, Pseudomonas* and *Pseudoxanthomonas* were found on all the five media in the present study thereby showing culture adaptability. Other genera viz., *Brevibacterium, Comamonas, Delftia* and *Yimella* are reported for the first time in the adult midgut of *An. stephensi.* The highest bacterial count of $2.4x10^5$ cfu/midgut and $15x10^5$cfu/midgut were obtained from the midgut of larvae and pupae of wild population on Blood Agar respectively. Similarly in another study, four media were utilized and among which Brain Heart agar and Nutrient Agar supported the maximum genera[19]. Dong et al., had observed highest TVC in the female midgut of *Anopheles gambiae* with an average of 40,000 cfu/midgut using Luria berthaniagar[9]. Several studies have been undertaken on midgut microbiota of *Anopheles* using different media which identified a few bacterial species as compared to the current study[15, 19, 21, 30–31]. Recovery of novel and additional bacterial genera using more number of media, for the first time in the current study, signifies the necessity to use multiple media in the identification of microflora of the midgut of *An. stephensi* using culture based technique.

Although, *Aeromonas, Brevibacterium, Lysinibacillus, Pseudomonas, Staphylococcus* and *Kocuria* have already been reported in *An. stephensi* but in different developmental stages and locations unlike here[4, 15–16, 28, 30]. In the present study, apart from those already reported, several new genera viz., *Delftia,Pedobacter* and *Micrococcus* were reported for the first time in *Anopheles stephensi*. However, these have been previously reported in *Aedes sp., Culex sp.* and *An. gambiae* which suggest that microbiota of different mosquito species inhabiting similar environmentscould be common as has been earlier suggested[9, 12, 15, 27, 32–34]. Out of the 21 genera reported in the current study, genus *Luteimicrobium* was identified for the first time in the mosquito gut. So far the presence of *Luteimicrobium*is reported from long horn beetle[24].In the present study, *Micrococcus* and *Bacillus* were the most frequently isolated bacteria from the two field populations of *An. stephensi*.

In case of lab population, except *Bacillus*, microbiota of all immature and adult stages and breeding water was distinct indicating its high variability. On the other hand, the presence of *Bacillus* and *Micrococcus* in breeding water, larvae, pupae and adult stages of *An. stephensi* in the two field populations signifies their transstadial transmission potential. The presence of different isolates of *Micrococcus* in a single clade of Phylogenetic tree further corroborates the above hypothesis (Fig. S5). However, an experimental validation using markers such as green fluorescent (GFP) is necessary to confirm this fact as has been done in case of *Asaia* species found in *Anopheles* gut[26]. Similarly, it is reported previously that due to an incomplete sterilization of *An. stephensi* gut, *Bacillus* could be retained in different developmental stages[15].

The presence of *Micrococcus* of Actinobacteria phylum in the wild population has been previously reported in several mosquito species such as *Culex quinquefasciatus, Aedes albopictus* and *Aedes aegypti*[4, 32, 34–35]. In contrast to the hypothesis of metamorphosis, which alters the classes of bacteria in immatures and adult mosquitoes[36-37], the presence of *Micrococcus* in all developmental stages provides an evidence that the metamorphosis, from one stage to another, does not completely eliminate gut microbiota. Similar observation was made in *Culex tarsalis* and *Culex pipiens* mosquitoes where some bacterial communities from the larval stages were shown to be sequestered in meconial peritrophic membrane and passed on to the adult stages[37–38]. In addition to passing through the different stages, *Micrococcus* also managed to escape the immune response of *Aedes aegypti* where it was melanised but not phagocytised[39].

Another bacterial genus *Leucobacter* was found to be present in all the developmental stages of populations of both the field samples. This presence of different *Leucobacter* isolates in different developmental stages in the same clade of phylogenetic tree with high similarity (Fig. S4-S5) provides further evidence to the possibility of transstadial transmission. However, experimental evidence is necessary to validate the hypothesis. It is noteworthy that in the earlier studies too, the genus *Leucobacter* was reported from the distinct stages of different mosquito species like in adult female of *Culex quinquefasciatus* and *Aedes albopictus*[32, 40], larvae of *Aedes. aegypti* and *Ae. albopictus*[34, 41] and adult males of *Ae. albopictus*[34].

Vector breeding site plays a significant role in microbiome acquisition in the midgut of mosquitoes[42]. In the present study, the microbiota of the three breeding sites of *Anopheles stephensi,* a major malaria vector in India, was also explored. While 15 genera were observed in field population, eleven were reported in the laboratory population. Additionally, the genera observed in the two field populations were found almost common (e.g. *Bacillus, Exiguobacterium, Leucobacter, Micrococcus* and *Pedobacter*), while different genera (*Brevibacterium., Delftia, Chryseobacterium, Pseudomonas, Elizabethkingia* and *Comamonas*) were observed in the laboratory population, the reason for which cannot be explained without more data and further experimental evidence is needed to corroborate this phenomenon of distinctiveness of microbiota between the field and laboratory cultures of *An. stephensi* immatures. A higher bacterial diversity was found in immatures reared in the breeding water than the laboratory populations reared in the tap water, suggesting that microbiota of the adults emerging from these two populations would also likely to be distinct. Similar observations have previously been made in *An. stephensi* population of Haryana, India[15]. It is pertinent to mention here that, all the protocols and conditions for maintaining the mosquito life cycle stages of both field and lab mosquitoes were similar. Differences in the water utilized for the rearing of mosquitoes can possibly be an important factor for the observed variation in the diversity of microbiota in field and lab population. A reduction of the relative abundance of microbiota in the tap water has also been previously demonstrated in *An. gambiae* in Africa[43].

There is significant evidence that the presence of microbiota in the mosquito population depends on several factors but their living environment plays a key role in shaping their microbiome[44]. The presence of different bacterial genera in different breeding waters has been observed in the present study as has also been observed in the earlier studies carried out across different states of India. However, the genera reported in present study from Goa are different from those reported from Haryana, Mangalore, Chennai and Bangalore populations except *Bacillus* and *Pseudomonas*[15, 28]. The ubiquitous presence of *Bacillus* across different geographical location indicates its versatility and diverse ecological adaptation ranging from saprophytic nature in the soil to symbiotic relationship with different insects[45]. This is mainly accomplished because many *Bacillus* species display varied physiological abilities which help them to thrive in almost every natural environment[46]. The statistical quantification of the bacterial microbiome would indicate their significance in the host.The diversity index quantifies bacterial diversity in the community and describes it numerically. In present study, both Simpsons diversity index and Shannon evenness were found to be low in one of the field breeding habitats (FS-2) of *An. stephensi,* even though 6 genera were present. This suggests uneven distribution of a single genus mainly due to the abundance of a single isolate. In contrast, high Shannon diversity was found in the female midgut of immatures of

the other field sample (FS-2). In the laboratory and FS-2 populations, Simpson's diversity was found to be low in the breeding habitat, larvae, pupae and female midgut except in the male midgut. This was due to the presence of larger number of genera in the male midgut. Shannon evenness is used to record how well the species are distributed among the individuals[23]. Interestingly, the Shannon evenness was found to be uniform in all the developmental stages of the field population obtained from FS1 sample, which further suggests that the transstadial transmission of some of the genera occurs. On the contrary, the Shannon evenness was highly fluctuating in the lab population, suggesting the uneven distribution of bacterial genera in the midgut of immatures from the lab samples.

CONCLUSION

In conclusion, to the best of our knowledge, this is the first approach in studying the culturable microbiome of the breeding habitat as well as population of immatures and adults of *An. stephensi*. The study emphasises the need for using multiple media to determine maximum diversity in gut microbiome. By analysing the phylogenetic tree and statistical data, it was concluded that diversity of microbiota is high in field populations as compared to laboratory population of *An. stephensi*. In this study, some bacterial isolates already reported in different mosquito species and beetle genera were identified for the first time in *An. stephensi.* Interestingly, the presence of identical *Micrococcus* and *Leucobacter* isolates in the field populations and different stages suggest that these genera might have adapted themselves to *An. stephensi* as symbionts. Further analysis is necessary to validate the above hypothesis. Further, elucidating the role of bacterial genera like *Bacillus, Micrococcus* and *Leucobacter* in *An. stephensi* will help in identifying suitable candidates for paratransgenesis. There is also need for further studies to identify the candidate microbes with stable transstadial traits so that they can be studied for their role in vector competence.

Conflict of interest: None

REFERENCES

1. World Health Organization. World malaria report 2019;1–10.
2. Kumar A, Sharma V, Thavaselvam D, Sumodan PK. Control of *Anopheles stephensi* breeding in construction sites and abandoned overhead tanks with *Bacillus thuringiensis* var. *israelensis*. J *Am Mosq Control Assoc* 1995; *11*(1): 86–9.
3. NVBDCP. National Framework for Malaria Elimination in India. 2016. *http://nvbdcp.gov.in/Doc/National-framework-for-malaria-elimination-in-India-2016–2030*. (Accessed on)
4. Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Pascale JM, *et al*. Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Negl Trop Dis* 2012; *6*(3): e1561.
5. Thomas S, Ravishankaran S, Justin JA, Asokan A, Mathai MT, Valecha N, *et al*. Overhead tank is the potential breeding habitat of *Anopheles stephensi* in an urban transmission setting of Chennai, India. *Malaria J* 2016; *15*(1): 274–3.
6. Kumar A, Thavaselvam D. Breeding habitats and their contribution to *Anopheles stephensi* in Panaji. *Indian J Malariol* 1992; *29*(1): 35–40.
7. Gimonneau G, Tchioffo MT, Abate L, Boissière A, *et al* . Composition of *Anopheles coluzzii* and *Anopheles gambiae* microbiota from larval to adult stages. *Infect Genet Evol* 2014; *28*: 715–24.
8. Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, *et al*. Midgut micro*biota* of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog* 2012; *8*(5): e1002742.
9. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* 2009; *5*(5): e1000423.
10. Fouda MA, Hassan MI, Al-Daly AG, Hammad KM. Effect of midgut bacteria of *Culex pipiens* L. on digestion and reproduction . *J Egypt Soc Parasitol* 2001; *31*(3) 767–780.
11. Dobson SL, Fox CW, Jiggins FM. The effect of *Wolbachia*-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. *Proc Biol Sci* 2002; *269*(1490): 437–45.
12. Tchioffo MT, Boissière A, Churcher TS, Abate L, Gimonneau G, Nsango SE, *et al*. Modulation of malaria infection in *Anopheles gambiae* mosquitoes exposed to natural midgut bacteria. *PLoS One* 2013; *8*(12): e81663.
13. Cappelli A, Damiani C, Mancini MV, Valzano M, Ramirez JL, Favia G. *Asaia* Activates Immune Genes in Mosquito Eliciting an Anti- *Plasmodium* Response : Implications in Malaria Control. *Front Genet* 2019 ; *10*: 836.
14. Rami A, Raz A, Zakeri S, Dinparast Djadid N. Isolation and identification of *Asaia sp*. in *Anopheles spp*. mosquitoes collected from Iranian malaria settings: Steps toward applying paratransgenic tools against malaria. *Parasit Vectors* 2018; *11*(1): 367.
15. Rani A, Sharma A, Rajagopal R, Adak T, Bhatnagar RK. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. *BMC Microbiol* 2009; *9*: 96.
16. Dinparast Djadid N, Jazayeri H, Raz A, Favia G, Ricci I, Zakeri S. Identification of the midgut microbiota of *An. stephensi* and *C. maculipennis* for their application as a paratransgenic tool against malaria. *PLoS One* 2011; *6*(12): e28484.
17. Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS One* 2011; *6*(9): e24767.
18. Chavshin AR, Oshaghi MA, Vatandoost H, Yakhchali B, Zarenejad F, Terenius O. Malpighian tubules are important determinants of *Pseudomonas* transstadial transmission and longtime persistence in *Anopheles stephensi*. *Parasit Vectors* 2015; *8*(1): 36.
19. Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A, Enayati AA, *et al*. Identification of bacterial micro-

flora in the midgut of the larvae and adult of wild caught *Anopheles stephensi*: A step toward finding suitable paratransgenesis candidates. *Acta Trop* 2012; *121*(2): 129–34.
20. Christophers SR. The Fauna of British India, Including Ceylon and Burma. Diptera. Family Culicidae. Francis T Vol. IV, Tribe Anophelini 1933; 371.
21. Lindh JM, Terenius O, Faye I. 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiaesensu la*to and *A. funestus mosquitoes* reveals new species related to known insect symbionts. *Appl Environ Microbiol* 2005; *71*(11): 7217–7223.
22. Sambrook J, Fritsch EF, Maniatis T, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, USA, 2nd edition, 1989; p.1546.
23. Möhlmann TWR, Wennergren U, Tälle M, Favia G, Damiani C, Bracchetti L, *et al*. Community analysis of the abundance and diversity of mosquito species (Diptera: Culicidae) in three European countries at different latitudes. *Parasit Vectors* 2017; *10*: 510.
24. Kim H, Oh HW, Kim JA, Park DS, Park HM, Bae KS. *Luteimicrobium xylanilyticum* sp. nov., isolated from the gut of a long-horned beetle, Massicus raddei. *Int J Syst Evol Microbiol* 2014; *64*: 1401–5.
25. Tchioffo MT, Boissière A, Churcher TS, Abate L, Gimonneau G, Nsango SE, *et al*. Modulation of malaria infection in *Anopheles gambiae* mosquitoes exposed to natural midgut bacteria. *PLoS One* 2013; *8*(12): e81663.
26. Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, *et al*. Bacteria of the genus Asaia stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *PNAS* 2007; *104*(21): 9047–9051.
27. Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LHR, Ravelonandro P, *et al*. Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiol Ecol* 2011; *75*(3): 377–89.
28. Kalappa DM, Subramani PA, Basavanna SK, Ghosh SK, Sundaramurthy V, Uragayala S, *et al*. Influence of midgut microbiota in *Anopheles stephensi* on *Plasmodium berghei* infections. *Malar J* 2018; *17*(1): 385.
29. Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, *et al*. Mosquito-Bacteria Symbiosis: The Case of *Anopheles gambiae* and *Asaia*. *Microb Ecol* 2010; *60*(3): 644–54.
30. Pumpuni CB, Demaio J, Kent M, Davis JR, Beier JC. Bacterial population dynamics in three anopheline species: The impact on *Plasmodium* sporogonic development. *Am J Trop Med Hyg* 1996; *54*(2): 214–218 .
31. Pidiyar VJ, Jangid K, Patole MS, Shouche YS. Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgut based on 16s ribosomal RNA gene analysis. *Am J Trop Med Hyg* 2004; *70*(6): 597–603.
32. Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN, Sukumaran D, *et al*. Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. *Plos one* 2013; *8*(11): e80453.
33. Charan SS, Pawar KD, Severson DW, Patole MS, Shouche YS. Comparative analysis of midgut bacterial communities of *Aedes aegypti* mosquito strains varying in vector competence to dengue virus. *Parasitol Res* 2013; *112*(7): 2627–37.
34. Yadav KK, Datta S, Naglot A, Bora A, Hmuaka V, Bhagyawant S, *et al*. Diversity of cultivable midgut microbiota at different stages of the asian tiger mosquito, *Aedes albopictu*s from Tezpur, India. *PLoS One* 2016; *11*(12): e0167409.
35. Audsley MD, Ye YH, McGraw EA. The microbiome composition of *Aedes aegypti* is not critical for *Wolbachia*-mediated inhibition of dengue virus. *PLoS Negl Trop Dis* 2017; *11*(3): e0005426.
36. Moncayo AC, Lerdthusnee K, Leon R, Robich RM, Romoser WS. Meconial peritrophic matrix structure, formation, and meconial degeneration in mosquito pupae/pharate adults: histological and ultrastructural aspects. *J Med Entomol* 2005; *42*(6): 939–944.
37. Moll RM, Romoser WS, Modrakowski MC, Moncayo AC, Lerdthusnee K. Meconial Peritrophic Membranes and the Fate of Midgut Bacteria During Mosquito (Diptera: Culicidae) Metamorphosis. *J Med Entomol* 2001; *38*(1): 29–32.
38. Duguma D, Hall MW, Rugman-Jones P, Stouthamer R, Terenius O, Neufeld JD, *et al*. Developmental succession of the microbiome of *Culex* mosquitoes Ecological and evolutionary microbiology. *BMC Microbiol* 2015; *15*: 140
39. Hillyer JF, Schmidt SL, Christensen BM. Rapid Phagocytosis and Melanization of Bacteria and *Plasmodium* Sporozoites by Hemocytes of the Mosquito *Aedes aegypti. J Parasitol* 2003; *89*(1): 62–9.
40. Muturi EJ, Dunlap C, Ramirez JL, Rooney AP, Kim CH. Host blood-meal source has a strong impact on gut microbiota of *Aedes aegypti. FEMS Microbiol Ecol* 2019; *95*(1).
41. Coon KL, Valzania L, McKinney DA, Vogel KJ, Brown MR, Strand MR. Bacteria-mediated hypoxia functions as a signal for mosquito development. *Proc Natl Acad Sci U S A* 2017; *114*(27): E5362–E5369.
42. Buck M, Nilsson LKJ, Brunius C, Dabiré RK, Hopkins R, Terenius O. Bacterial associations reveal spatial population dynamics in *Anopheles gambiae* mosquitoes. *Sci Rep* 2016; *6*(1): 22806
43. Akorli J, Namaali PA, Ametsi GW, Egyirifa RK, Pels NAP. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (sensu lato) during colonization in the laboratory. *Parasit Vectors* 2019; *12*: 27.
44. Guégan M, Zouache K, Démichel C, Minard G, Van VT, Potier P, *et al*. The mosquito holobiont : fresh insight into mosquito-microbiota interactions. *Microbiome* 2018; *6*: 49.
45. Ceuppens S, Boon N, Uyttendaele M. Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiol Ecol* 2013; *84*(3): 433–50.
46. Turnbull PCB. *Bacillus*. In: Baron S, editor. Barron's Medical Microbiology. 4th ed. University of Texas Medical Branch; Galveston, TX, USA: 1996.

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