Iranian Journal of Microbiology
Volume 15 Number 2 (April 2023) 258-266

ORIGINAL ARTICLE

Isolation and identification of microbiota of *Culex quinquefasciatus* for their application as paratransgenic tools in vector control

Leila Nourani, Abbasali Raz, Navid Dinparast Djadid*

Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran

Received: June 2022**, Accepted:** February 2023

ABSTRACT

Background and Objectives: Although the study on the bacteria residing in the mid-gut, salivary gland, and reproductive organs of insect vectors have drawn appeal to the host-pathogen interactions, we know comparatively less about microbiota that naturally exist in different mosquito organs within Iran.

Materials and Methods: In the current investigation, PCR assay by using 16S rRNA gene amplification and DNA sequencing, in addition to the traditional culture-based approach utilized for the detection of cultivable bacterial assemblages in mid-gut and reproductive tracts of *Culex quinquefasciatus.*

Results: The identified bacteria isolated from different tissues of 45 individuals were consisted of *Achromobacter, Aeromonas, Arthrobacter, Asaia, Enterobacter, Gluconobacter, Klebsiella, Lysinibacillus, Micrococcus, Psuedomonas* and *Serratia*. The results showed that Proteobacteria was the most prevalent phylum in both genders' mid-gut and reproductive tracts, and *Asaia* was the most common bacteria that originated in adult females and males' tissues.

Conclusion: These outcomes recommend that the discovered microbiome may span through *Cx. quinquefasciatus* populations. This data can be utilized to interfere with the transmission of pathogens and design new strategies for the control of mosquito-borne diseases.

Keywords: Vector-borne diseases; *Culex*; Polymerase chain reaction; Bacteria

INTRODUCTION

Blood feeding by southern house mosquito, *Culex quinquefasciatus* Say 1823 (Diptera: Culicidae) causes zoonotic diseases like West Nile fever, lymphatic filariasis (*Wuchereria bancrofti*), St. Louis encephalitis, Western equine encephalitis, Japanese encephalitis (1-7), and also introduced as a potential vector for Zika virus with major influences on public health (8). The transmission of avian malaria, *Plasmodium* spp., has also been indicated by this anthropophilic vector (9, 10).

Diseases transmission is attributed to the assortment of microbiota that directly and indirectly has a great impact on mosquitoes' populations (11). The microbiota has been introduced as principal co-existent organisms with pronounced impressions on mosquitos' characteristics related to re-production success, the fitness of female adults, immunity versus pathogens, sensitivity and resistance to pesticides, persistence, and control of different populations. As the mosquitoes protect the symbiotic microbiota, they contribute to nutritional and ecological supports which affect vector competence and devel-

*Corresponding author: Navid Dinparast Djadid, Ph.D, Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran. Tel: +98-21-64112464 Fax: +98-21-66480749 Email: navidmvrg@gmail.com

opment, from immature stages to the adult, in return (12, 13). The prominence of microbial communities is due to the three-way relationship between the vector, the bacteria, and the disease pathogen (11). The major consequences of insecticide resistance, sustainability, and organizational complications were accompanied by the lack of treatments and vaccines. The application of conventional vector control strategies like personal protective procedures and control of mosquito populations served as the limited methods to prevent arboviral diseases (14, 15). Due to the medical significance of human vector-borne diseases, a large amount of cost and time are dedicated to developing novel vaccines and drugs to control pathogens. Application of paratrangenic method by using resident bacteria in mosquitoes may set out as a promising technique toward control strategies (16). This method would be useful to generate paratransgenic bacteria along with symbiotic organisms living inside different organs of mosquito species. This strategy facilitates quickly spreading of a special gene within wild mosquito populations. Besides, the capability of paratransgenic bacterial strains by expressing a protein that targets various species, could be applied versus a verity of competent vectors (17-19). Following researches on *Culex* species' microbiota have shown the bacterial strains of different phylum and classes (20-25).

Although the study on the bacteria residing in mid-guts, salivary glands, and reproductive tracts of insect' vectors have recently increased considerably, few investigations have studied the bacterial assemblage in reproductive tracts and mid-gut of Culicidae species in Iran, mostly concentrated on *Anopheles* (26-28). Toward this goal, we aimed to identify bacteria diversity originated in field-collected larvae and laboratory-reared *Cx. quinquefasciatus* to find if bacterial collection differs over the different tissues: mid-gut, ovary, and testes, and to report common bacterial strains in mosquito organs to define gender- or organ-specific bacteria.

MATERIALS AND METHODS

Samples collection and identification. To detect the cultivable bacteria in mid-gut and reproductive tracts of *Cx. quinquefasciatus*, 45 individuals were examined in a PCR-based study. The specimens were male and female' adults collected from urban locations with semi mountainous or hilly places in Iranshahr, Sistan and Baluchestan province, south-east of Iran. Larvae were collected by the standard dipping technique and transferred to the lab settled in the National Insectarium of Malaria and Vector Research Group (MVRG) at Pasteur Institute of Iran (Karaj, Alborz province). The captured larvae were retained in pre-sterilized cages until maturation. Laboratory-reared colonies of adult female and male mosquitoes were used for the current study. The morphological identification of adult mosquitoes was carried out by the standard taxonomic key of Culicinae, for *Culex* species (29). This study was carried out following the guidelines and protocols permitted by the Research Committee and Institutional Ethics Committee of National Institute for Medical Research Development, Iran (No: IR.NIMAD.REC.1397.012).

Sample preparation and bacteriological experiments. To examine the microbial diversity, mid-guts, testes, and ovary pairs of blood-fed adult mosquitoes were separated. Prior to the dissection, mosquitoes were anesthetized for two minutes at -20°C and then transferred on the ice. The samples surface was sterilized by washing the whole body in the micro-tubes (2 ml) containing 70% ethanol and shaking gently for several minutes (21). Under sterile settings (biological laminar flow hood class-II), the microscopically dissections were performed individually and then, separated organs were preserved in 200 µl sterile physiological saline (0.9% NaCl). The suspension was then homogenized by an electrical homogenizer and 100 µl of that were inoculated into the 5 ml cultural tubes containing LB broth media for general bacteria and handmade enhancement media for *Asaia* sp. growth (30). After overnight incubation (for general bacteria) and three days incubation (for *Asaia*), the bacterial growth was checked by turbidity observation. Subsequently the suspension of the grown up bacteria was cultured on LB agar, MacConkey agar and *Asaia* enriched agar plates, followed by incubation at 30°C for 12 to 16 hours for *Asaia* and 37°C for general bacteria. The purified single bacterial colonies were obtained after several sub-cultures.

DNA isolation of pure colonies, PCR, and sequencing. The bacterial DNA extractions were performed by DNA isolation kit (Yekta Tajhiz Azma Inc., Tehran, Iran) following the manufacturer's instructions. The quality of isolated DNA was quan-

tified to approve the adequate genetic material for sequencing by using Nanodrop spectrophotometer. Molecular identification of pure colonies was performed by amplification of 1,450 base pairs fragments of the 16S rRNA gene by the universal primers of 16suF: 5'-GAGTTTGATCCTGGCTCAG-3' and 16suR: 5'-GTTACCTTGTTACGACTT-3'(27). Moreover, the specific primers Asafor (5'-GCG CGT AGG CGG TTT ACA C-3') and Asarev (5'-AGCGTCAG-TAATGAGCCAGGT T-3') was used for confirmation of *Asaia* (30). Reaction mixtures were prepared using 12.5 µl PCR red master-mix (AMPLIQON, Denmark), 0.5 µm of each primer, 100 ng of the extracted DNA as a template and ddH2O up to final volume of 25 µl. Reactions were performed at 94°C for 5 min and cycled 35 times through a protocol of 30 s at 94°C, 30 s at 60°C, and 100 s at 72°C. Finally, the reactions were maintained at 72°C for 10 min. Positive PCR products were confirmed by agarose gel electrophoresis (1%) and GreenViewer DNA stain (Parstous Biotechnology, Mashhad, Iran) visualized by UV transilluminator. Positive amplicons were purified and sequenced bi-directly using automated DNA sequencer ABI 3500 (Applied Biosystems Inc., CA, USA) in Codon Genetic Group (Tehran, Iran).

Phylogenetic analysis and haplotype network. The attained sequences were edited, assembled, and aligned using MAFFT online algorithm and BioEdit v.7. To ascertain the nearest related strains, the bacterial sequences were blasted via nucleotide BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nearest taxon to each sequence was used in phylogenetic tree analysis. The sequences divergence was measured with Kimura 2-parameter distance matrix, implemented in MEGA6.0 using the pairwise comparison of all sequences (31). Phylogenic analysis accomplished by Bayesian inference executed in MrBayes v3.2 software (32) with two concurrent MCMC searches for 1,000,000 generations and sampled in each 1,000 generations. The posterior probabilities were calculated at the end of the analysis, when the burn-in period of 50% was set and the chains were reached stationary status under evolutionary model selected by ModelTest v.7 software (33). The resultant phylogenetic tree was edited and visualized by FigTree v1.4. In addition, haplotype diversity was estimated using DnaSP v.5 package (34) and haplotype network was also constructed to determine the genetic distances among detected *Asaia* isolates in Iran and other published species by using Median-Joining method in PopART program.

RESULTS

To define bacterial diversity in mid-gut and reproductive tracts, two sets of primers for general bacteria and *Asaia* were used in molecular approach. The general primers amplify a ~ 1400 base-pairs (bp) fragment of 16S rRNA gene whereas the *Asaia*-specific primers set to amplify a ~180 bp. Successful PCR results approved *Asaia* presence in different specimens on the agarose gel electrophoresis. Positive amplicons produced by general and specific primer sets were sequenced. The analysis of sequences showed that the identified bacteria isolated from mid-guts and reproductive tracts of 45 individuals of *Cx. quinquefasciatus* included 11 bacterial genera; *Achromobacter, Aeromonas, Arthrobacter, Asaia, Enterobacter, Gluconobacter, Klebsiella, Lysinibacillus, Micrococcus, Pseudomonas*, and *Serratia* (Table 1). The common and specific bacterial genus in each organ is shown in Fig. 1.

The results showed that Proteobacteria was the most prevalent phylum in both genders' mid-gut and reproductive tracts, and Actinobacteria and Firmicutes were less dominant phyla. *Lysinibacillus*, a genus belonging to Firmicutes found in males' mid-gut. *Arthrobacter* and *Micrococcus* which belong to Actinobacteria were recorded in females' mid-gut.

The bacterial genera *Asaia, Klebsiella*, and *Serratia* were detected in ovaries while *Asaia, Gluconobacter, Klebsiella, Pseudomonas*, and *Serratia* were isolated form testis. *Asaia, Klebsiella*, and *Serratia* were similarly identified bacteria in both genders' reproductive tracts. Females and males' mid-gut were mainly colonized by *Asaia* and *Enterobacter*. Correspondingly to other organs *Serratia* was found in the mid-gut of both genders. Moreover, other detected taxa comprising *Aeromonas, Arthrobacter* and *Micrococcus* in females' mid-gut whereas *Achromobacter* and *Lysinibacillus* were found in males' mid-gut. Although the diversity of bacterial taxa changed in the mosquito organs, the most common bacteria that originated from adult female and male mosquitoes was *Asaia* which found in mid-gut and reproductive tracts of both genders (Table 1 and Fig. 1).

The amplified and edited sequences (n=27) were submitted into GenBank database under accession num-

Table 1. Isolated and identified bacteria of *Cx. quinquefasciatus* from Iran.

Bacteria genera	Phyla	Nearest taxon from BLASTn (Similarity %)	No. of sequence (s)	MF	OF	MM	TM
Achromobacter	*Proteobacteria*	*A. xylosoxidans* (99.93%)	1			√	
Aeromonas	*Proteobacteria*	*A. hydrophila* (99.86%)	2	√			
Arthrobacter	*Actinobacteria*	*A. agilis* (99.93%)	1	√			
Asaia	*Proteobacteria*	*A. bogorensis* (99.78%)	12	√	√	√	√
Enterobacter	*Proteobacteria*	*E. hormaechei* (99.86%)	2	√		√	
Gluconobacter	*Proteobacteria*	*Gluconobacter* sp. (99.85%)	1				√
Klebsiella	*Proteobacteria*	*K. michiganensis* (99.93%)	2		√		√
Lysinibacillus	*Firmicutes*	*L. fusiformis* (99.86%)	1			√	
Micrococcus	*Actinobacteria*	*M. luteus* (99.91%)	1	√			
Pseudomonas	*Proteobacteria*	*P. mosselii* (99.86%)	1				√
Serratia	*Proteobacteria*	*S. marcescens* (99.68%)	3		√	√	√

Bacteria isolated of each organ are summarized as mid-gut bacteria of females (MF), mid-gut bacteria of males (MM), ovary' bacteria (OF), testis' bacteria (TM).

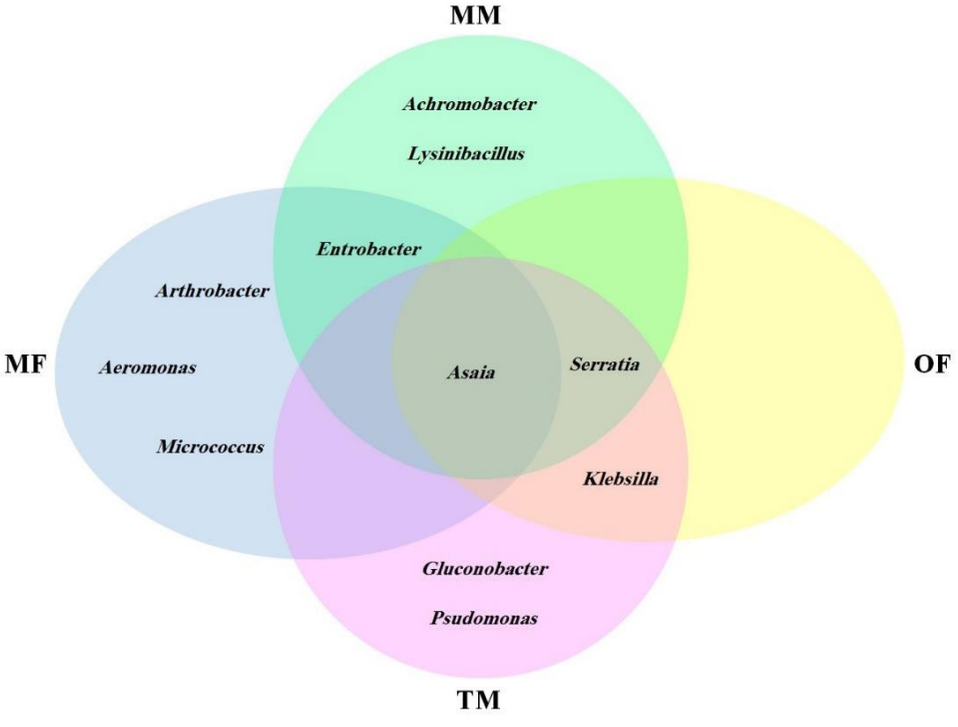

Fig. 1. Detected bacteria isolated from *Cx. quinquefasciatus* organs. MF: mid-gut bacteria of female mosquitoes, MM: mid-gut bacteria of male mosquitoes, OF: ovary- and TM: testis originated bacteria are shown by different colors.

bers of OP734286 to OP734312. In the subsequent stage, these sequences were aligned using ClastalW with a retrieved dataset from GenBank (n=66) to infer the phylogenetic relationship of detected species. The Bayesian tree was assembled by using the retrieved data from previous investigations around the world from NCBI beside attained sequences based on ~1400 bp of the 16S rRNA gene presented the placement of isolates clustered along with the nearest taxon (Fig. 2). *Enterobacter* and *Klebsiella* were positioned as the earliest sister taxa while *Serratia, Pseudomonas* and *Aeromonas* were clustered in parapyletic situations.

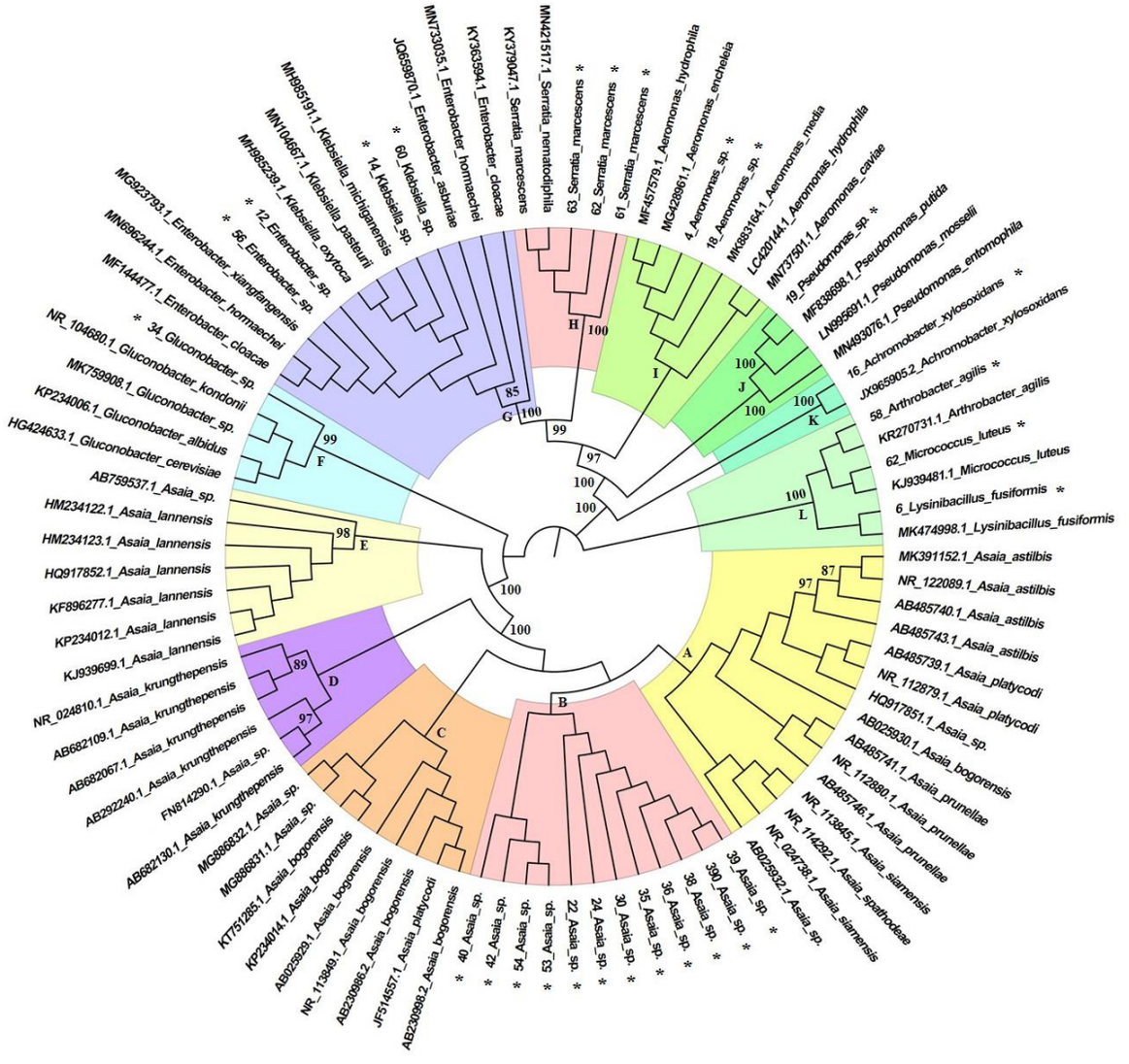

Fig. 2. The phylogenetic Bayesian tree of detected bacteria in *Cx. quinquefasciatus* collected from South-eastern Iran and retrieved sequences of nearest taxon in blast analysis. Detected bacteria in the current study are specified with star.

The position of *Asaia* bacterial species in the related cluster showed that the detected samples of this study are grouped with *A. bogorensis* whereas the other species clustered in the paraphyletic situations. *Gluconobacter* was the nearest taxon to *Asaia* has placed as the sister taxa. Besides, the genus of *Micrococcus* was placed as the out-group in the basal clade (Fig. 2). Blast analysis of sequences showed that two *Klebsiella* sequences originated from different organs, the testis, and ovary which are grouped near *K. michiganensis.*

The median joining haplotype network was constructed to screen the genetic relationship of *Asaia* haplotypes. The results revealed the presence of 18 haplotypes (haplotype diversity ± SD: 0.9056 ± 0.047) in 49 sequences (Fig. 3). Iranian isolates placed in haplotypes (Hap 1) near Hap 2, 8, 17 which are similar to position in the Bayesian tree (more information for haplotypes are given in appebdix). Hap 1 was comprised of 12 sequences of *Asaia* isolated from females mid-gut (n=7), male mid-gut (n=2) and testis (n=1) and ovaries (n=2). Hap 2 was included 6 sequences, *A. bogorensis* (n=4), *Asaia* sp. (n=1), *A. platycodi* (n=1) while Hap 3 was comprised *A. bogorensis* (n=1). The other haplotypes belonged *Asaia* sp. (Hap 4, accession number: AB025932.1), *A. krungthepensis* (Hap 5, NR113845.1) and *Asaia* sp. (Hap 5, NR114292.1), *Asaia* sp. (Hap 6, MG886832.1), *A. bogorensis* (Hap 7, NR113849.1), *A. krungth-*

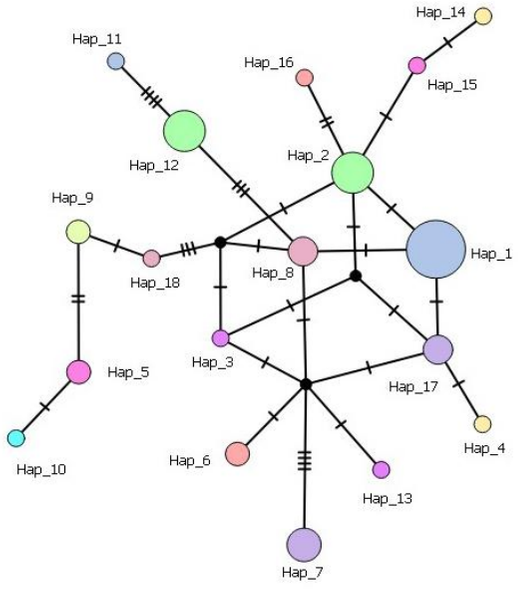

Fig. 3. *Asaia* species haplotype network estimated and illustrated by DNaSP package and PopART software. The median-joining method was used to draw the haplotype network and the size of the circles specifies the haplotype frequency. The mutational steps between haplotypes are demonstrated by lines.

epensis (Hap 8, NR024810.1), *Asaia* sp. (Hap 9, MG886831.1), *A. platycodi* (Hap 10, NR112879.1), *A. lannensis* (Hap 11, KF896277.1), *A. astilbis* (Hap 12, AB485743.1 and NR122089.1) and *A. prunellae* (Hap 13, AB485741.1). Genetic distances of Iranian *Asaia* isolates and global species demonstrated a range of differences from 0.1% to 1.1% in pairwise distances analysis.

DISCUSSION

The mosquito's organs are involved in interactions with pathogens and other microbial communities, having a possible influence on their host biology and vector competence for transmission of particular diseases (22, 35). In this study, we characterized the bacteria hosted in mid-gut and reproductive tracts of *Cx. quinquefasciatus*, a recognized hematophagous vector for human viral diseases, collected from the southeast of Iran, by using the traditional culture-based method, PCR method and DNA sequencing (36). Our analysis of the mid-gut and reproductive tracts' bacterial colonies disclosed the presence of 11 genera with no organized pattern either shared by one or both genders or present in different organs. Nonetheless, other investigation introduced Actinobacteria, as the dominant phylum in *Cx. quinquefasciatus* (37), in the present study, Proteobacteria was the prevalent phylum discovered in different organs. Among all bacteria discovered, *Asaia* was the common genus in both reproductive tracts and mid-gut, and in both genders. A study to investigate the mid-gut microbiota of female *Cx. quinquefasciatus* mosquitoes captured from India by using the application 16S ribosomal DNA sequencing from cultivable bacteria showed the occurrence of 83 bacterial species and 31 genera with the dominant incidence of Proteobacteria, followed by Firmicutes and Actinobacteria (22). To the best of our knowledge, this is the first study of the identification and isolation of different organs' microbiota in *Cx. quinquefasciatus* from the southeast of Iran. *Staphylococcus* and *Enterobacter* possessed the highest rate of the detected genus in *Culex* species, represented in the most sampling field locations (22). Other investigations proved the presence of a new *Aeromonas* species isolated form *Cx. quinquefasciatus*, and *Sphingomonas* sp. was detected in two species, *Culex restuans* and *Cx. pipiens* (21, 38). The existence of midgut microbe's flora demonstrated seasonal changes in various groups of *Cx. quinquefasciatus* at the genus level. The genera *Wolbachia*, *Pantoea*, *Acinetobacter*, *Pseudomonas*, and *Staphylococcus* were of dominant bacteria in inspected groups (39).

The reported data for bacterial community inhabited in *Cx. quinquefasciatus*' organs in both adults and larvae, and different genders at the global level, demonstrated the variety of bacterial genera and species, such as the following prevalent species: *Asaia*, *Aeromonas*, *Acinetobacter*, *Bacillus*, *Escherichia*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Serratia*, *Staphylococcus* and *Wolbachia* and less common bacteria. The earlier researches have shown that bacteria were isolated from various such as the mid-gut, testis, ovary, saliva gland, abdomen or whole mosquitoes, and in different age groups (larvae, pupae and adult) with the more concentrate of researches on mid-gut. In the current study, the microbiota originated from mid-gut and productive organs of males and females mosquitoes. Other investigations have collected *Culex* species samples were wild adults (wild) or larvae collected from the field and reared in the insectary. The given results in the present study were based on collected larvae samples reared in insectary which

did not show any pattern in accordance with latter experiments, biased for wild or insectary-reared hosts.

The previous investigations on *Culex* species microbiota were run by conventional bacterial culture, PCR 16S rRNA, and DNA sequencing for the detection of various bacterial strains (40, 41) and continued by up-to-date approaches: RNA shotgun metagenomic sequencing, next generation sequencing, culturomics, and MALDI-TOF MS (24, 42-44). Next-generation sequencing method revealed the presence of *Wolbachia*, *Streptococcus*, *Stenotrophomonas*, *Staphylococcus*, *Corynebacterium*, and *Rothia* in ovaries of female *Cx. quinquefasciatus* and *Ochrobacterum* in the testis of similar host species (24) whereas *Elizabethkingia* sp. and *Pantoea dispersa* were isolated from ovaries in the same host, isolated by PCR 16S rRNA and DNA sequencing (40). Though the main target for detection of microbiota was mid-gut, some other investigations focused on defining bacterial assemblage of salivary gland. These bacteria included *Asaia*, *Cupriavidus*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Staphylococcus*, and *Veillonella* identified by next-generation sequencing in *Cx. quinquefasciatus* (24).

Most researchers isolated bacteria from one or more specific tissues and the others performed examinations in whole mosquitoes' culture. Their results demonstrated the detection of *Actinobacillus* in *Cx. pipiens* by RNA shotgun metagenomic sequencing (45) and *Acinetobacter* in *Cx. pipiens*/*restuans* and *Cx. salinarius* by 16S rRNA Illumina sequencing method (46). Furthermore, *Anaplasma*, *Asaia*, *Bacillus*, *Bradyrhyzobium*, *Escherichia*, *Shigella*, *Gluconobacter*, *Moraxella*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Rickettsiella*, *Spiroplasma*, and *Wolbachiaare* were discovered from whole body cultures in other researches (45-47).

Researches based on the role of microbial communities in mosquito biology and pathogen intervention may lead to the development of novel vector control methods by using alterations of the microbiota (48-51) among the detected bacteria in this investigation, *Asaia*, *Enterobacter*, *Pseudomonas*, and *Serratia* have been recommended as prospective nominees for vector control strategies through paratransgenic manipulations (24), and as an appropriate method based on the procedure to modify symbiotic species for transportation of effector molecules to wild vectors (24, 26-28). In this sense, well established and cultivable microorganisms in mosquitoes are appropriate for genetic modifications with the ability to transmit anticipated qualities to the next generations, pass from males to females during mating, and colonize in reproductive organs and salivary glands (30, 52). There are numerous studies that investigate the origin of these bacteria transferring into hosts. Horizontal transferring of bacteria occurred through breeding water contamination in both larvae and adults that cause bacterial existence in mid-gut (53). In another investigation, *Vagococcus fluvialis* was detected in the mid-gut of *Cx. quinquefasciatus* was common species isolated from domestic animals and human resources which verified the hypothesis of the food origin of the mosquito microbiota (23).

CONCLUSION

In this investigation, the composition of bacterial communities of reproductive tracts and mid-gut of *Cx. quinquefasciatus* mosquito was studied in southeastern Iran. We have recognized several bacteria which may be utilized in mosquito control approaches. Novel data attained by using culture-dependent and culture-independent approaches to the microbiota diversity in various geographical regions will help scientists predict the possible circulation of microbiota.

ACKNOWLEDGEMENTS

The authors would like to announce their truthful gratitude to who supported in the sampling and lab experiments. Current research was supported by Elite Researcher Grant Committee under award number 971531 from the National Institutes for Medical Research Development (NIMAD), Tehran, Iran. We also express our sincere gratitude to S. Yaghoubi and M.R. Nourani for their support during our investigations.

REFERENCES

1. Amraoui F, Krida G, Bouattour A, Rhim A, Daaboub J, Harrat Z, et al. *Culex pipiens*, an experimental efficient vector of West Nile and Rift Valley fever viruses in the Maghreb region. *PLoS One* 2012; 7(5): e36757.

2. Hamer GL, Kitron UD, Brawn JD, Loss SR, Ruiz MO, Goldberg TL, et al. *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *J Med Entomol* 2008; 45: 125-128.
3. Turell MJ. Members of the *Culex pipiens* complex as vectors of viruses. *J Am Mosq Control Assoc* 2012; 28(4 Suppl): 123-126.
4. Diaz LA, Flores FS, Beranek M, Rivarola ME, Almirón WR, Contigiani MS. Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Cordoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332-334.
5. Samy AM, Elaagip AH, Kenawy MA, Ayres CF, Peterson AT, Soliman DE. Climate change influences on the global potential distribution of the mosquito *Culex quinquefasciatus*, vector of West Nile virus and lymphatic filariasis. *PloS One* 2016; 11(10): e0163863.
6. Bakhshi H, Mousson L, Vazeille M, Zakeri S, Raz A, De Lamballerie X, et al. High transmission potential of West Nile Virus Lineage 1 for *Cx. pipiens* s.l. of Iran. *Viruses* 2020; 12: 397.
7. Bakhshi H, Beck C, Lecollinet S, Monier M, Mousson L, Zakeri S, et al. Serological evidence of West Nile virus infection among birds and horses in some geographical locations of Iran. *Vet Med Sci* 2021; 7: 204-209.
8. Liu Z, Zhou T, Lai Z, Zhang Z, Jia Z, Zhou G, et al. Competence of *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes as Zika virus vectors, China. *Emerg Infect Dis* 2017; 23: 1085-1091.
9. Pruck-Ngern M, Pattaradilokrat S, Chumpolbanchorn K, Pimnon S, Narkpinit S, Harnyuttanakorn P, et al. Effects of artesunate treatment on *Plasmodium gallinaceum* transmission in the vectors *Aedes aegypti* and *Culex quinquefasciatus*. *Vet Parasitol* 2015; 207: 161-165.
10. Nourani L, Zakeri S, Dinparast Djadid N. Dynamics of prevalence and distribution pattern of avian *Plasmodium* species and its vectors in diverse zoogeographical areas-A review. *Infect Genet Evol* 2020; 81: 104244.
11. Weiss B, Aksoy S. Microbiome influences on insect host vector competence. *Trends Parasitol* 2011; 27: 514-522.
12. Weiss BL, Wang J, Aksoy S. Tsetse immune system maturation requires the presence of obligate symbionts in larvae. *PLoS Biol* 2011; 9(5): e1000619.
13. Dillon RJ, Dillon VM. The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* 2004; 49: 71-92.
14. Scolari F, Casiraghi M, Bonizzoni M. Aedes spp. and their microbiota: a review. *Front Microbiol* 2019; 10: 2036.
15. McCarroll L, Paton MG, Karunaratne SH, Jayasuryia HT, Kalpage KS, Hemingway J. Insecticides and mosquito-borne disease. *Nature* 2000; 407: 961-962.
16. Wilke ABB, Marrelli MT. Paratransgenesis: a promising new strategy for mosquito vector control. *Parasit Vectors* 2015; 8: 342.
17. Raharimalala FN, Boukraa S, Bawin T, Boyer S, Francis F. Molecular detection of six (endo-) symbiotic bacteria in Belgian mosquitoes: first step towards the selection of appropriate paratransgenesis candidates. *Parasitol Res* 2016; 115: 1391-1399.
18. Wang S, Ghosh AK, Bongio N, Stebbings KA, Lampe DJ, Jacobs-Lorena M. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc Natl Acad Sci U S A* 2012; 109: 12734-12739.
19. Welch M, Govindarajan S, Ness JE, Villalobos A, Gurney A, Minshull J, et al. Design parameters to control synthetic gene expression in *Escherichia coli*. *PLoS One* 2009; 4(9): e7002.
20. Pidiyar V, Kaznowski A, Narayan NB, Patole M, Shouche YS. *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. *Int J Syst Evol Microbiol* 2002; 52: 1723-1728.
21. 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: 597-603.
22. 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.
23. Chandel K, Parikh RY, Mendki MJ, Shouche YS, Veer V. Isolation and characterization of *Vagococcus* sp from midgut of *Culex quinquefasciatus* (Say) mosquito. *J Vector Borne Dis* 2015; 52: 52-57.
24. Mancini MV, Damiani C, Accoti A, Tallarita M, Nunzi E, Cappelli A, et al. Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. *BMC Microbiol* 2018; 18: 126.
25. Juma EO, Allan BF, Kim C-H, Stone C, Dunlap C, Muturi EJ. Effect of life stage and pesticide exposure on the gut microbiota of *Aedes albopictus* and *Culex pipiens* L. *Sci Rep* 2020; 10: 9489.
26. Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A, Enayati AA, et al. Identification of bacterial microflora in the midgut of the larvae and adult of wild caught *Anopheles stephensi:* a step toward finding suitable paratransgenesis candidates. *Acta Trop* 2012; 121: 129-134.
27. Dinparast Djadid N, Jazayeri H, Raz A, Favia G, Ricci I, Zakeri S. Identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* for their application as a paratransgenic tool against malaria. *PloS One* 2011; 6(12): e28484.
28. Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand

MR, Raeisi A, Terenius O. Isolation and identification of culturable bacteria from wild *Anopheles culicifacies*, a first step in a paratransgenesis approach. *Parasit Vectors* 2014; 7: 419.
29. Dehghan H, Sadraei J, Moosa-Kazemi SH, Abolghasemi E, Solimani H, Jaffari Nodoshan A, et al. A pictorial key for *Culex pipiens* complex (Diptera: Culicidae) in Iran. *J Arthropod Borne Dis* 2016; 10: 291-302.
30. 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. *Proc Natl Acad Sci U S A* 2007; 104: 9047-9051.
31. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013; 30: 2725-2729.
32. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003; 19: 1572-1574.
33. Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mol Biol Evol* 2020; 37: 291-294.
34. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009; 25: 1451-1452.
35. Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasit Vectors* 2013; 6: 146.
36. Farajollahi A, Fonseca DM, Kramer LD, Marm Kilpatrick A. "Bird biting" mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. *Infect Genet Evol* 2011; 11: 1577-1585.
37. Coon KL, Brown MR, Strand MR. Gut bacteria differentially affect egg production in the anautogenous mosquito *Aedes aegypti* and facultatively autogenous mosquito *Aedes atropalpus* (Diptera: Culicidae). *Parasit Vectors* 2016; 9: 375.
38. Muturi EJ, Kim C-H, Bara J, Bach EM, Siddappaji MH. *Culex pipiens* and *Culex restuans* mosquitoes harbor distinct microbiota dominated by few bacterial taxa. *Parasit Vectors* 2016; 9: 18.
39. Suo P, Wang K, Yu H, Fu X, An L, Bhowmick B, et al. Seasonal variation of midgut bacterial diversity in *Culex quinquefasciatus* populations in Haikou City, Hainan Province, China. *Biology (Basel)* 2022; 11: 1166.
40. Telang A, Skinner J, Nemitz RZ, McClure AM. Metagenome and culture-based methods reveal Candidate bacterial mutualists in the Southern House Mosquito (Diptera: Culicidae). *J Med Entomol* 2018; 55: 1170-1181.
41. Adly E, Hegazy AA, Kamal M, Abu-Hussien SH. Midguts of *Culex pipiens* L. (Diptera: Culicidae) as a potential source of raw milk contamination with pathogens. *Sci Rep* 2022; 12: 13183.
42. Gazzoni Araujo Gonçalves G, Feitosa APS, Portela-Júnior NC, De Oliveira CMF, De Lima Filho JL, Brayner FA, et al. Use of MALDI-TOF MS to identify the culturable midgut microbiota of laboratory and wild mosquitoes. *Acta Trop* 2019; 200: 105174.
43. Ramos-Nino ME, Fitzpatrick DM, Eckstrom KM, Tighe S, Hattaway LM, Hsueh AN, et al. Metagenomic analysis of *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes from Grenada, West Indies. *PLoS One* 2020; 15(4): e0231047.
44. Tandina F, Almeras L, Koné AK, Doumbo OK, Raoult D, Parola P. Use of MALDI-TOF MS and culturomics to identify mosquitoes and their midgut microbiota. *Parasit Vectors* 2016; 9: 495.
45. Chandler JA, Liu RM, Bennett SN. RNA shotgun metagenomic sequencing of northern California (USA) mosquitoes uncovers viruses, bacteria, and fungi. *Front Microbiol* 2015; 6: 185.
46. Novakova E, Woodhams DC, Rodríguez-Ruano SM, Brucker RM, Leff JW, Maharaj A, et al. Mosquito microbiome dynamics, a background for prevalence and seasonality of West Nile virus. *Front Microbiol* 2017; 8: 526.
47. Osei-Poku J, Mbogo CM, Palmer WJ, Jiggins FM. Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Mol Ecol* 2012; 21: 5138-5150.
48. Ricci I, Damiani C, Capone A, DeFreece C, Rossi P, Favia G. Mosquito/microbiota interactions: from complex relationships to biotechnological perspectives. *Curr Opin Microbiol* 2012; 15: 278-284.
49. Duguma D, Hall MW, Rugman-Jones P, Stouthamer R, Terenius O, Neufeld JD, et al. Developmental succession of the microbiome of *Culex* mosquitoes. *BMC Microbiol* 2015; 15: 140.
50. Duguma D, Hall MW, Smartt CT, Neufeld JD. Temporal variations of microbiota associated with the immature stages of two Florida *Culex* mosquito vectors. *Microb Ecol* 2017; 74: 979-989.
51. Duguma D, Hall MW, Smartt CT, Neufeld JD. Effects of organic amendments on microbiota associated with the *Culex nigripalpus* mosquito vector of the Saint Louis Encephalitis and West Nile viruses. *mSphere* 2017; 2(1): e00387-16.
52. De Freece C, Damiani C, Valzano M, D'amelio S, Cappelli A, Ricci I, et al. Detection and isolation of the α-proteobacterium *Asaia* in *Culex* mosquitoes. *Med Vet Entomol* 2014; 28: 438-442.
53. Coon KL, Vogel KJ, Brown MR, Strand MR. Mosquitoes rely on their gut microbiota for development. *Mol Ecol* 2014; 23: 2727-2739.