

Original Article

Metagenomics Characterization of *Ixodes ricinus* Intestinal Microbiota as Major Vector of Tick-Borne Diseases in Domestic Animals

Manijeh Yousefi-Behzadi^{1,2}, Atefeh Mehrabi³, Mozghan Ahmadinezhad¹, Mahdi Rohani⁴, Saied Reza Naddaf⁵, Amin Bagheri⁶, Masoomeh Shams-Ghahfarokhi⁷, Ebrahim Maghsoudlou⁸, Nooshin Mojahed⁹, Leila Mounesan¹, Zahra Tahmasebi^{1,2}, Aria Sohrabi^{1,2}, Mostafa Salehi-Vaziri¹⁰, Zahra Salehi¹¹, *Mehdi Razzaghi-Abyaneh¹¹

¹Department of Epidemiology and Biostatistics, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Tehran, Iran

²National Reference Laboratory of Plague, Tularemia and Q Fever, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Akanlu, Kabudar-Ahang, Hamadan, Iran

³School of Advanced Medical Sciences, Islamic Azad University, Tehran Medical Branch, Tehran, Iran

⁴Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran

⁵Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran

⁶Department of Pathobiology, Faculty of Veterinary Medicine, Science and Research, Tehran, Iran

⁷Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

⁸Department of Computer Science, School of Computing, Southern Illinois University Carbondale, Illinois

⁹Department of Physiology, School of Medicine, Southern Illinois University, Carbondale, Illinois

¹⁰Department of Arboviruses and Viral Hemorrhagic Fevers, Pasteur Institute of Iran, Tehran, Iran

¹¹Department of Mycology, Pasteur Institute of Iran, Tehran, Iran

*Corresponding author: Dr Mehdi Razzaghi-Abyaneh, E-mails: mrab442@yahoo.com, mrab442@pasteur.ac.ir

(Received 14 Feb 2023; accepted 11 Jun 2023)

Abstract

Background: Understanding the microbiota of disease vectors can help for developing new strategies to prevent the transmission of vector pathogens. *Ixodes ricinus* is one of the most notorious tick vectors with increasing importance in Iran and other parts of the world while there is limited data on its microbiota. This study aimed to use metagenomics for identifying the *I. ricinus* tick's microbiota of Iran.

Methods: A total of 39 adult ticks were collected from Mazandaran (21 females), Gilan (17 females), and Golestan (1 male). Five tick pools prepared from 39 adults of *I. ricinus* were subjected to metagenomics analysis. The data were analyzed by targeting the V6 region of the 16S rRNA gene by Illumina 4000 Hiseq sequencing.

Results: Among hundreds of intestinal microbiota identified by metagenomics, various pathogenic microorganisms distributed in 30 genera and species including those responsible for tick-borne diseases resided in the genera *Coxiella*, *Rickettsia*, and *Burkholderia* were found.

Conclusion: Our results indicated that metagenomics identifies bacteria genera and species which cannot be easily recognized by routine methods. The presence of such pathogenic bacteria indicates the importance of possible zoonotic diseases in this region which could affect public health. These results further substantiate the importance of advanced metagenomics analyses to identify neglected tick-borne pathogens which enable researchers to provide efficient mapping roads for the management of emerging and re-emerging infectious diseases.

Keywords: Tick-borne disease; Microbiota; *Ixodes ricinus*; Next-generation sequencing; Emerging pathogens

Introduction

Ticks are worldwide ectoparasites belonging to the Arachnida class, superorder parasitiformes

with over 900 species categorized into two families Ixodidae (hard ticks) and Argasidae (soft

ticks) (1). Among over 700 hard ticks, *Ixodes ricinus* is one of the most abundant species infesting various hosts, including birds, mammals, and occasionally reptiles which derived from *Acarus* species (2, 3). They meet their nutritional requirements by feeding on the vertebrate's blood hosts and ecological and anthropogenic factors affect *I. ricinus* ticks' population and activity (4). Since *I. ricinus* ticks prefer a warm and humid environment, the temperature and the moisture amount in the air and soil are determinants (5); however, these ticks also occur in areas such as Iceland (6), which has a sub-polar oceanic climate with poor vegetation (7). The availability and the abundance of suitable hosts are other fundamental components. Nevertheless, geographical regions with lowlands, forests, and green pastures are excellent habitats for *I. ricinus* ticks geographical regions with lowlands, forests, and green pastures are excellent habitats for *I. ricinus* ticks (8). Moreover, agriculture-associated activities, biotope alteration, insecticides, and anti-tick vaccination for tick management are considered human-induced elements that impact the frequency of ticks (9). The microbiome (microbiota) is a group of diverse microorganisms present inside and outside another organism (10, 11). They can be symbiotic, commensal, or even pathogenic for the host. The tick microbiome includes different species of bacteria, viruses, and protozoa, some of which are called tick-borne pathogens (12, 13). Ticks carry a variety of microbiota inside their salivary glands, midgut, and ovary, and they can transmit them to their hosts (human or animal) during blood-feeding (14). *Ixodes ricinus* ticks are widely known as vectors of Tick-borne disease (TBDs) pathogens causing medical and veterinary conditions (15). Lyme disease and tick-borne encephalitis (TBE) are caused by *Borrelia burgdorferi* sensu lato and the tick-borne encephalitis virus (TBEV), both harbored by *I. ricinus* (16). Some of the pathogens previously identified to be carried by *I. ricinus* are *Babesia divergens* (17), *Babesia microti*, *Babesia venatorum* (18), *Anaplasma*

phagocytophilum (17), *Rickettsia Helvetica* (19), *Rickettsia slovaca*, *Rickettsia monacensis* (20), *Borrelia miyamotoi* (21), *Ehrlichia phagocytophila* (17), Louping ill virus (22), *Bartonella henselae* (23), *Coxiella burnetii* (24), and *Francisella tularensis* (25). Non-pathogenic microorganisms are also present in the tick's microbiome, including those involved in amino acid and lipid metabolism pathways (26). Metagenomics is the science of studying the genetic content of environmental samples. The next-generation sequencing method plays a vital role in analyzing DNA and RNA materials. This high-throughput technology provides a reliable and accurate way of identifying the microorganisms in almost any microbial community (11, 27). The V6 region of the 16S rRNA gene is highly conserved among different bacterial species and can provide reliable high resolution taxonomic classification of bacteria and archaea. This region is also relatively short and easy to amplify and to sequence using PCR-based methods, making it easier and more cost-effective compared to longer regions for large-scale metagenomic studies (27). The present study investigated the bacteria in the midgut of *I. ricinus* ticks collected from the Golestan, Mazandaran, and Gilan provinces in the northern part of Iran. This littoral region of the Caspian Sea is covered by the Hyrcanian forests creating a suitable environment for ticks' activity and reproduction regarding the humidity, temperature, and a vast range of suitable hosts. It is consequential to improve our knowledge about the pathogens in ticks to organize the best and most effective tick management strategies protecting both humans and animals (whether domestic or wild) against TBDs.

Materials and Methods

Study area and population

The study area in the Caspian Sea littoral of Iran included the southern border of the Caspian Sea and the northern heights of Alborz mountains, covering an area of 58,167 Km² in three provinces of Gilan (37°04'27"N 50°12' 35"E)

Mazandaran (36.2262° N, 52.5319° E), and Golestan (37.2898° N, 55.1376° E). The Hyrcanian forest in northern Iran covers 3,400,000 hectares on the Alborz Mountains' northern slopes stretching down to the Caspian Sea coastal areas (Fig. 1). General characteristics of the climate are too much rain in all seasons, especially in autumn and winter, relatively high humidity in all seasons, and low-temperature difference during the day due to moisture.

Sampling

Ticks were collected from domestic animals, i.e., cattle and sheep, in autumn from 6 to 11 Nov. 2021 during the period of tick multiplication. The *I. ricinus* ticks were transferred to the laboratory immediately under ambient, live conditions and identified under stereomicroscope using available taxonomic key for ticks (28). A total number of 39 *I. ricinus* ticks isolated from cattle and sheep were collected in 5 localities (Table 1) (Fig. 1). The collected ticks were from different locations in the coastal area in the north of Iran. Only one male tick was among 39 collected ticks, considered a separate pool. Furthermore, this was from Golestan-Barfatan where no female tick was found. More data, such as location, number of ticks, and hosts, are available in Table 1.

Separation of midgut from *Ixodes ricinus*

To make sure no microorganism contamination from the ticks' surface or the environment enters our study, before doing any operation, the ticks were washed carefully in 70% alcohol for 3 minutes and then rinsed with sterile water alternatively three times. First, the ticks identified in the laboratory were placed individually on a slide, a few drops of molten paraffin were poured on them to keep the ticks fixed on the slide, and the paraffin was allowed to harden. The ticks were dissected under an entomological loop using a small pair of ophthalmic scissors. The ticks were dissected under an entomological loop using a small pair of ophthalmic scissors. The dorsal scutum and the white grape clusters-like salivary glands were

completely removed, then the whole intestinal section was separated and transferred to a sterile opener with some distilled water inside and then stored in a -20 °C freezer for DNA extraction steps.

DNA Extraction

DNA of isolated intestines was extracted using a genomic DNA extraction kit (Bio Basic, Canada according to the manufacturer's instructions. Isolates were disrupted by adding 200 mg of glass beads (0.2 mm diameter) Plus 400 µL of lysis buffer (containing 200 mM Tris-HCL, pH= 8, 25 mM EDTA, SDS 0.5% W/V, NaCl 250 mM) and vortexed using a Micro smash MS-100R (Tomy Seiko, Japan) at 4000 rpm for the 60 s. Then added 200 µL buffer PP was to microtubes containing the above mixture and incubated at -20 °C for 5 minutes. Finally, DNA was extracted using the chloroform and isopropanol methods (29).

Metagenomics analysis

Genomic DNA was extracted from five pools prepared based on five locations (each pool contained the collected ticks of that location). The final product, a mix of microorganisms' DNA content, was sent to MacroGen Company (S. Korea) for sequencing. The DNA was amplified for V6 region of the 16S rRNA gene by Herculase II Fusion DNA Polymerase kit using 341F forward primer and 850-R reverse primer (primer sequence (CCTACGGGNGG CWGCAG) and (GACTACHVGGGTATCT AATCC), respectively. The library preparation was done by DNA Polymerase Nextera XT kit, which is suitable for working with small genomes. The product was sequenced with a MiSeq device, and it was set to sequence the PCR product 300 base pairs from both ends. The MiSeq benchtop sequencer output is over 100,000 reads with a length of 300 and a total of more than 30 million bases. Then analysis was performed on the output Fastq file after checking the quality.

NGS data analysis

The first step in NGS data analysis is checking the quality of the raw fastq files by Fastqc and MultiQC (30, 31). It is notable that for 16 S amplicon sequencing, having many repeats in the samples is very common. DADA2 software was used to de-noise and join the forward and reverse reads (32). Trimming lengths for forward and reverse reads are very important in this step. Since the primers used were 341F and 850R, the expected length of amplicons is $850-341=509$, so with paired-end read length equal to 300, we have $2*300-509=91$ bases overlap without trimming. It is recommended to leave 20 + natural amplicon variance bases for overlap and trim the rest for best results. In checking with Fastqc results, the trim length for forward reads was set at 300 (no trimming) and for reverse reads at 250 (trimming 50 bases from the end). Since the length of the target region (509) is much higher than the read length (300), the risk of having an adapter sequence at the end of forward reads is negligible. Then the resulting OTUs were annotated with the SILVA (33) database assigning each sequence to the nearest species that share at least 90 percent of the sequence. QIIME 2 (34) platform has been utilized for visualization of data.

There are different methods for constructing a phylogenetic tree. The Maximum Likelihood (ML), which is the method we have used in this study infers a phylogenetic tree by calculating the likelihood of observing the given DNA or protein sequences under different evolutionary models and tree topologies. The tree with the highest likelihood is considered the most likely. ML is a powerful method that can incorporate complex models of sequence evolution, but it can be computationally intensive and may require substantial computational resources (35).

Results

Thirty-nine ticks were collected from three neighboring provinces of Gilan, Mazandaran, and Golestan in the north of Iran, creating five

pools based on the localities (Fig. 1). Following DNA extraction of each pool and amplification of V6 region of the 16S rRNA gene, sequence reads of 98042 (MOT11311), 114951 (MOT15054), 103101 (MOT15055), 68754 (MOT15056), and 112262 (MOT15061) were obtained from sequencing by Miseq Illumina. A large number of bacteria and the archaea phylum Woesearchaeota were observed in the tick microbiome community data. Because the total read count of Woesearchaeota was only 14, so we considered it low confidence and removed it from our data focusing only on bacteria. Also, there were 275 unassigned reads. The dominant taxonomy levels of phylum, class, order, family, genus, and species belonged to *Proteobacteria*, *Alphaproteobacteria*, *Rickettsiales*, *Rickettsiales Incertae sedis*, *Candidatus midichloria*, and *Burkholderia* sp. A3 (2016), while the minimum belonged to *Fusobacteria*, *Holophagae*, *Holophagae*. Subgroup 7, *Nocardiaceae*, *Curtobacterium*, and *Curtobacterium* sp. SW30, respectively.

Fourteen phyla were found in the five pools together (Figs. 2 and 3). Phylogenetic relationship of microbiota from *I. ricinus* intestine prepared by metagenomics analyses is shown in Fig.

Following Proteobacteria with over 93% frequency, Bacteroidetes, Actinobacteria, and Firmicutes were the most frequent phyla consisting of over 1% of the reads, and they exist in all pools despite low abundance in some. The rest (6% of the reads) were the reads with no related bacteria identified or bacteria with low read counts that were removed.

The analysis showed a difference in the relative abundance of the pool appointed to the male tick with the female's pool. The Armatimonadetes, Ignavibacteriae, Fusobacteria, and Saccharibacteria phyla were seen in the male tick microbiota but not in females. In contrast, Deinococcus-Thermus and Spirochaetae were only observed in females. Furthermore, the dominant phyla were different in the male sample. For example, the Actinobacteria phylum is far more frequent in the only male tick analyzed.

Fig. 3 shows the relative abundance of each sample in 4-6 of taxonomy levels (level 4: order, level 5: family, level 6: genus). The abundance of the top 30 detected bacteria with the most frequencies can be found in Table 2. Our results showed that pathogenic bacteria in the Rickettsia genera are the predominant pathogenic organisms in our collected ticks which

could be representative of the higher presence of rickettsiosis in the region of study. Indeed, the presence of *Burkholderia*, *Corynebacterium*, *Coxiella* and *Pseudomonas* in our samples showed the probable importance of neglected diseases but no significant difference was observed between sampling locations.

Table 1. Detailed information of tick sampling in the north of Iran

Sample ID	Province (Village)	Location	Number of tick(s)	Gender	Host
MOT1131	Golestan-Barfatan	36°53'05"N 54°47'54"E	1	Male	Sheep
MOT15054	Mazandaran (Klardasht-Banafsheh Deh)	36°30'00"N 51°15'00"E	11	Female	Cattle
MOT15061	Mazandaran (Chamestan-Joorband)	36°26'26"N 52°07'16"E	8	Female	Sheep
MOT15055	Mazandaran (Salman Shahr-Sisara)	36°42'12"N 51°13'50"E	2	Female	Cattle
MOT15056	Gilan (Narenjbon)	37°04'27"N 50°12'35"E	17	Female	Cattle

Table 2. Metagenomics data of relative abundance of top 30 identified bacteria at genus and species level in five pooled samples of *Ixodes ricinus* based on genus and species

Bacteria	RA *	Bacteria	RA*
<i>Candidatus midichloria</i>	0.69380803	<i>Burkholderia</i> sp. A3(2016)	0.058673062
<i>Rickettsia</i>	0.093340712	<i>Ralstonia</i> sp. 14I	0.012101088
<i>Ralstonia</i>	0.058673062	<i>alpha proteobacterium</i> Gsoil 1161	0.005054418
<i>Sediminibacterium</i>	0.012101088	<i>Bradyrhizobium elkanii</i>	0.003320421
<i>Bradyrhizobium</i>	0.008091988	<i>Bradyrhizobium</i> sp. CCBAU 51357	0.001586423
<i>Nitrobacter</i>	0.006222714	<i>Staphylococcus chromogenes</i>	0.001463445
<i>Aquabacterium</i>	0.006161225	<i>Pseudomonas putida</i>	0.001180594
uncultured	0.00538646	<i>Wautersiella</i> sp. MBG55	0.001119105
uncultured	0.004968333	<i>Pseudomonas putida</i> W15Oct28	0.000836254
<i>Mesorhizobium</i>	0.004722376	uncultured <i>Deinococci bacterium</i>	0.000823956
<i>Burkholderia-Paraburkholderia</i>	0.004710078	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i>	0.000737871
<i>Reyranella</i>	0.004316547	<i>Zea luxurians</i>	0.000737871
<i>Acinetobacter</i>	0.002803911	uncultured <i>Streptococcus</i> sp.	0.000725573
<i>Shewanella</i>	0.002607145	<i>Lactococcus lactis</i>	0.000700978
<i>Pseudomonas</i>	0.00252106	wastewater metagenome	0.000676382
<i>Corynebacterium1</i>	0.002299699	<i>Pseudomonas</i> sp. HOT12	0.000676382
<i>Staphylococcus</i>	0.002152124	<i>bacterium#1 from Psoroptes ovis</i>	0.000627191
<i>Sulfuritalea</i>	0.002102933	<i>Paracoccus</i> sp. B-1012	0.000565701
<i>Sphingobacterium</i>	0.001807785	uncultured <i>Methylocella</i> sp.	0.000553403
uncultured	0.001733997	<i>Shewanella</i> sp. AK55	0.000541106
<i>Citrobacter</i>	0.001623317	<i>Staphylococcus</i> sp. NR 4-12	0.00051651
<i>Coxiella</i>	0.001611019	<i>Sphingobacterium multivorum</i>	0.00051651
<i>Enhydrobacter</i>	0.001537232	<i>Stenotrophomonas maltophilia</i>	0.000430425
<i>Rhizomicrobium</i>	0.001475742	<i>Sediminibacterium</i> sp. OTC15	0.000405829
<i>Streptococcus</i>	0.001426551	<i>Nevskia terrae</i>	0.000405829
<i>Nevskia</i>	0.001401955	<i>Staphylococcus</i> sp. J33	0.000393531
<i>Bacillus</i>	0.001266679	<i>Caulobacter</i> sp. UR 6-06	0.000356638

Table 2. Continued ...

<i>Delfia</i>	0.001266679	<i>Comamonas aquatica</i>	0.00034434
<i>Empedobacter</i>	0.001168296	<i>Bacterium R78</i>	0.00034434
<i>Sorangium</i>	0.001155998	<i>Microbacterium laevaniformans</i>	0.000319744

*Relative Abundance

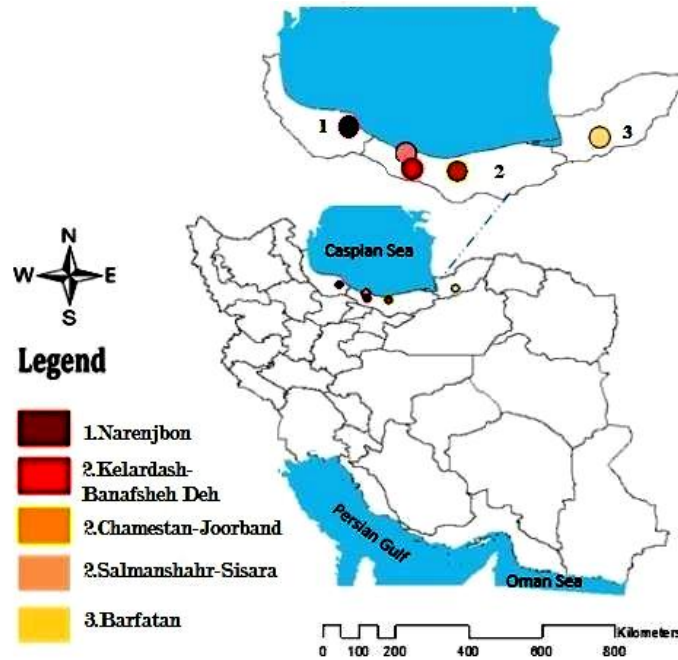


Fig. 1. Geographic distribution of *Ixodes ricinus* sampels in Gilan (No. 1), Mazandaran (No. 2) and Golestan (No. 3) provinces in the north of Iran using ArcGIS software. Esri

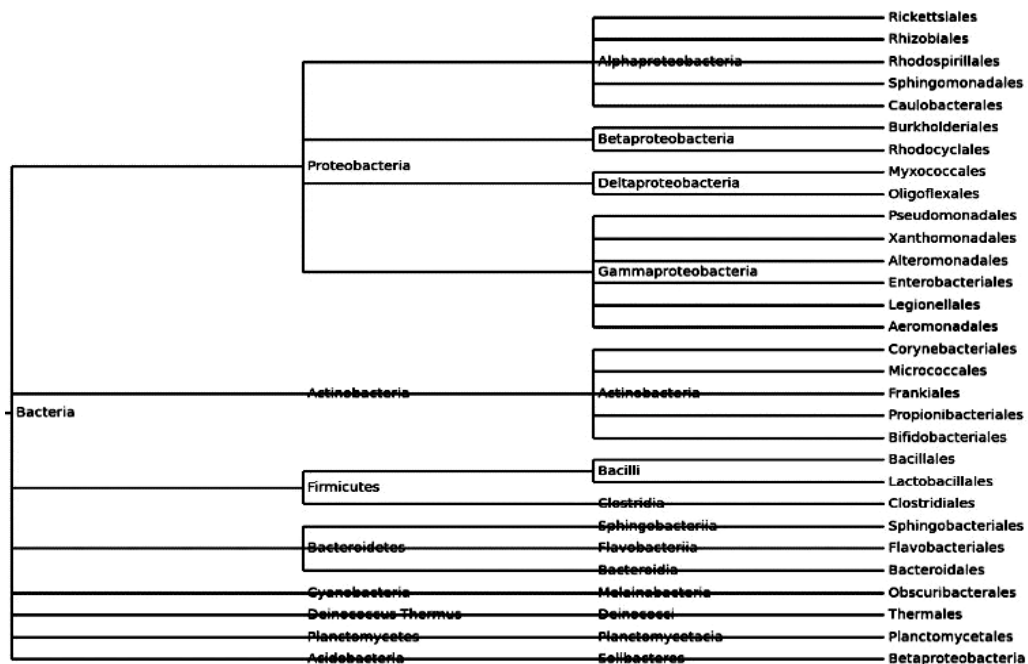


Fig. 2. Phylogenetic relationship of microbiota from *I. ricinus* intestine prepared by metagenomics analyses in 4 taxonomy levels of domain, phylum, class, and order using Biopython 1.81, Phylo library

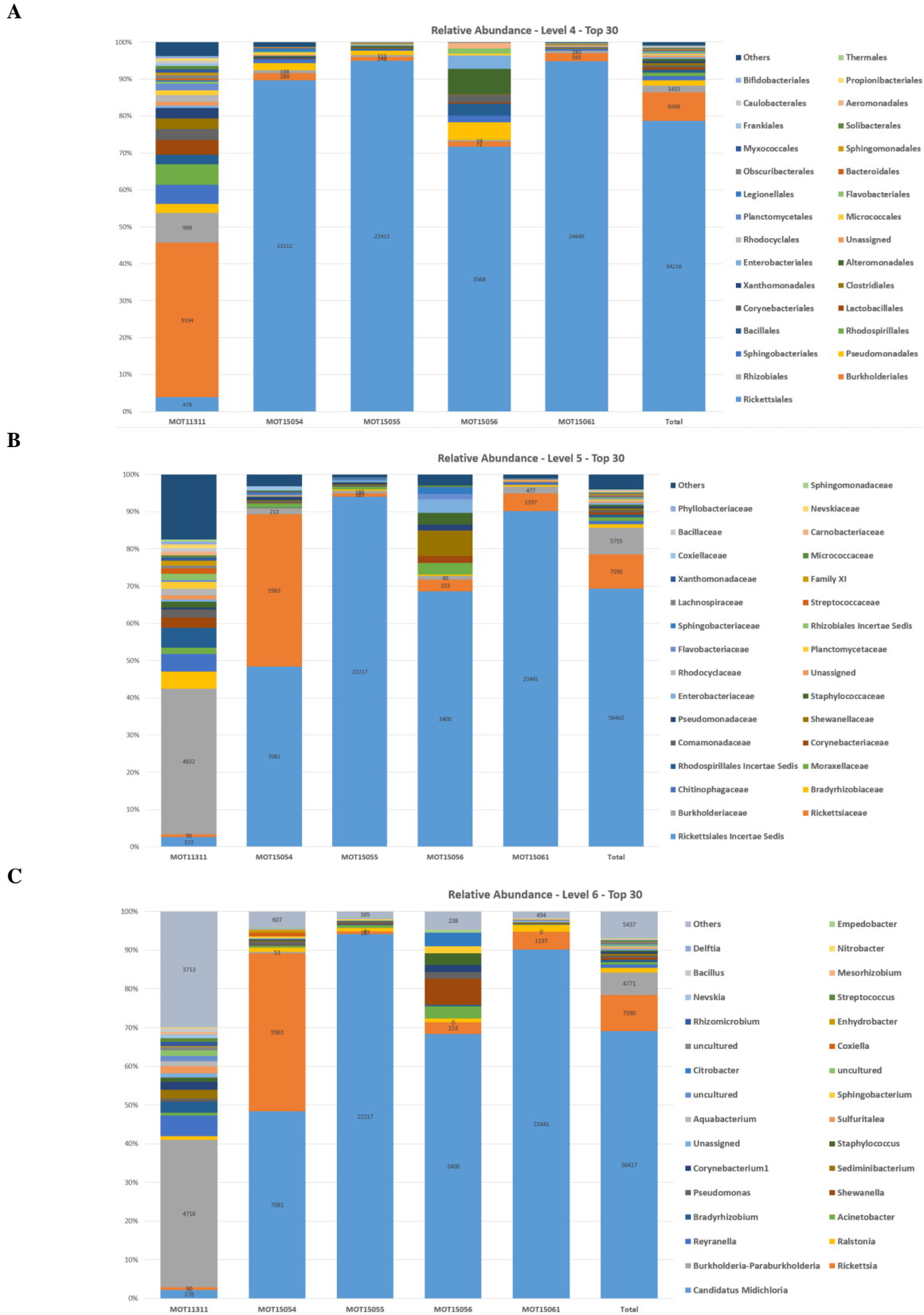


Fig. 3. Metagenomics data of relative abundance of top 30 bacteria found in five sample pools of *Ixodes ricinus* (A= order, B= family, C= genus) using Matplotlib 3.7.1 library

Discussion

This study was conducted to reveal and identification of midgut microbiota of *I. ricinus* ticks and their impact on disease distribution in 3 Northern provinces of Iran. Our data exhibited 14 phyla, of which Bacteria, Proteobacteria, Actinobacteria, and Firmicutes were the most abundant. In our study, Proteobacteria were the most abundant, which was consistent with a study in Italy and Spain (36, 37). In a study conducted in Iran on *Hyalomma anatolicum* tick, Proteobacteria was also the most abundant (38).

The present study also investigated the relative abundance of bacterial species in the *I. ricinus* tick. Among the different bacteria examined, 30 were more abundant in 5 pools of ticks. Similar to the present results, Bacteria, Actinobacteria, and Firmicutes were the most abundant after Proteobacteria, which accounted for more than 0.01% of the readings (39). Interestingly, in the study in Italy and Slovakia (36, 40), Actinobacteria were collected as one of the dominant groups in *I. ricinus* ticks and were much more abundant than the results of our research. According to the findings of previous studies, genus of *Candidatus midichloria* (0.69), *Rickettsia* (0.09), *Ralstonia* (0.05), and species of *Burkholderia* sp. (0.05), *Ralstonia* sp. 14I (0.01), alpha-proteobacterium Gsoil 1161 (0.00) was found with the highest frequency.

The results of our study were consistent with those of Spain (37) that *Candidatus midichloria* the most abundant bacteria in *I. ricinus* ticks. The high abundance of this bacterium may indicate the endosymbiosis of *Candidatus midichloria* mitochondrii (CMM)(41), whose high abundance in *I. ricinus* material is associated with its unique ability to localize inside mitochondria in the tick ovary. Because this endosymbiont is consistently detected in oocytes, CMM is thought to be maternally inherited (42). The results of the present study were consistent with the previous studies, so that in Italy and Slovakia, the *Rickettsia* genus was associated with large amounts in the collected *I. ricinus* ticks (36, 40). Also, another study identified a

close relationship between some tick symbionts and the potential presence of pathogenic *Rickettsia* or a parasitoid in ticks (43). In a study in Iran, *Rickettsia* was observed in the intestine of *Hyalomma anatolicum* tick (38). Another pathogen observed in the results of our study was *Coxiella*, which was not found in previous studies (39). This pathogen is the cause of Q fever, which is one of the common diseases between humans and animals. This disease affects many populations including human and animals every year and carries a high disease burden (44). *Coxiella* (45), *Rickettsia* (46), flavivirus (47), Crimean-Congo hemorrhagic fever (48), and *Burkholderia* (49) are among major health challenges impacting human and animals in Iran. As these diseases are zoonoses, they cause a significant disease burden for health systems every year (50).

Also, our data showed sex-dependent differences in microbiota in ticks. Unfortunately, due to financial constraints, we could not collect more ticks and only one male tick was observed in our study. The only male tick analyzed showed significantly higher bacterial richness and greater overall diversity than its females. A less diverse microbiota in female ticks has also been previously reported for some other tick species and in previous studies, the sample size of ticks was similar to our study (51, 52). However, in a study in America in 2021, female microbiota showed a significantly higher bacterial richness in one of the areas. Still, in general, it had less diversity than its male counterparts (53). The current study can provide preliminary data for further investigations. We also used the new metagenomics method to study and identify the tick's midgut, which showed a significant difference in the different types of pathogenic bacteria. Finally, this study observed zoonotic pathogens in the tick's intestine, indicating the importance of zoonotic diseases in the region.

Conclusion

In this study, the frequency of pathogenic bacteria in ticks was higher in males than that of females. Metagenomics enabled us to identify bacteria such as *Coxiella*, *Rickettsia* and *Burkholderia* which cannot be easily recognized by routine methods. The presence of such pathogenic bacteria indicates the importance of possible zoonotic diseases in this region which could affect the public health meaningfully. Overall, these results further substantiate the importance of advanced metagenomics analyses to identify neglected tick-borne diseases which provide mapping roads for management of emerging and re-emerging infectious diseases.

Acknowledgements

This work is supported by the Pasteur Institute of Iran (Code TP-9353). The authors would like to thank the Pasteur Institute of Iran for their cooperation during the study. Due to the importance of the study, the participants in this research were included in the list of authors.

Ethical considerations

There was no human and animal study that needed ethical concerns.

Conflict of interest statement

The authors declare there is no conflict of interests.

References

1. Walter DE, Proctor HC (1999) Mites: ecology, evolution and behaviour.
2. Sharifdini M, Norouzi B, Azari-Hamidian S, Karamzadeh N (2021) The first record of ectoparasites of raccoons (*Procyon lotor*) (Carnivora, Procyonidae) in Iran. *Persian J Acarol.* 10(1): 41–54.
3. Paules CI, Marston HD, Bloom ME, Fauci AS (2018) Tickborne Diseases-Confronting a Growing Threat. *N Engl J Med.* pmid: 30044925.
4. Černý J, Lynn G, Hrnková J, Golovchenko M, Rudenko N, Grubhoffer L (2020) Management options for *Ixodes ricinus*-associated pathogens: a review of prevention strategies. *Int J Environ Res Publ Health.* 17(6): 1830.
5. James M, Bowman A, Forbes K, Lewis F, McLeod J, Gilbert L (2013) Environmental determinants of *Ixodes ricinus* ticks and the incidence of *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in Scotland. *Parasitology.* 140(2): 237–246.
6. Alfredsson M, Olafsson E, Eydal M, Unnsteinsdottir ER, Hansford K, Wint W, Alexander N, Medlock JM (2017) Surveillance of *Ixodes ricinus* ticks (Acari: Ixodidae) in Iceland. *Parasit Vectors.* 10(1): 466.
7. Mooney PA, Rechid D, Davin EL, Katragkou E, de Noblet-Ducoudré N, Breil M, Cardoso RM, Daloz AS, Hoffmann P, Lima DCA, Meier R, Soares PMM, Sofiadis G, Strada S, Strandberg G, Toelle MH, Lund MT (2022) Land–atmosphere interactions in sub-polar and alpine climates in the CORDEX Flagship Pilot Study Land Use and Climate Across Scales (LUCAS) models–Part 2: The role of changing vegetation, *The Cryosphere.* 16: 1383–1397.
8. Bellato A, Pintore MD, Catelan D, Pautasso A, Torina A, Rizzo F, Mandola ML, Mannelli A, Casalone C, Tomassone L (2021) Risk of tick-borne zoonoses in urban green areas: A case study from Turin, northwestern Italy. *Urban Forest Urban Green.* 64: 127297.
9. Choubdar N, Oshaghi MA, Rafinejad J, Pourmand MR, Maleki-Ravasan N, Salehi-Vaziri M, Telmadarraiy Z, Karimian F, Koosha M, Rahimi-Foroushani A, Masoomi S, Arzamani K, Nejati J, Karami M,

- Mozaffari E, Salim-Abadi Y, Moradi-Asl E, Taghilou B, Shirani M (2019) Effect of meteorological factors on *Hyalomma* species composition and their host preference, seasonal prevalence and infection status to crimean-congo haemorrhagic fever in Iran. *J Arthropod Borne Dis.* 13 (3): 268–283.
10. Seedorf H, Griffin NW, Ridaura VK, Reyes A, Cheng J, Rey FE, Smith MI, Simon GM, Scheffrahn RH, Woebken D, Spormann AM, Van Treuren W, Ursell LK, Pirrung M, Robbins-Pianka A, Cantarel BL, Lombard V, Henrissat B, Knight R, Gordon JI (2014) Bacteria from diverse habitats colonize and compete in the mouse gut. *Cell.* 159(2): 253–266.
 11. Eybpoosh S, Haghdoost AA, Mostafavi E, Bahrampour A, Azadmanesh K, Zolala F (2017) Molecular epidemiology of infectious diseases. *Electron Phys.* 9(8): 5149.
 12. Lange A (2021) Interactions between intestinal commensal bacteria and different hosts: two sides of the same story: PhD Thesis, Universität Tübingen.
 13. Tabasi M, Eybpoosh S, Siadat SD, El-yasinia F, Soroush A, Bouzari S (2021) Modulation of the gut microbiota and serum biomarkers after laparoscopic sleeve gastrectomy: a 1-year follow-up study. *Obes Surg.* 31: 1949–1956.
 14. Narasimhan S, Fikrig E (2015) Tick microbiome: the force within. *Trends Parasitol.* 31(7): 315–323.
 15. Dantas-Torres F, Chomel BB, Otranto D (2021) Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol.* 28(10): 437–446.
 16. Wilhelmsson P, Jaenson TG, Olsen B, Waldenström J, Lindgren P-E (2020) Migratory birds as disseminators of ticks and the tick-borne pathogens *Borrelia* bacteria and tick-borne encephalitis (TBE) virus: a seasonal study at Ottenby Bird Observatory in South-eastern Sweden. *Parasit Vectors.* 13: 1–17.
 17. Rizzoli A, Silaghi C, Obiegala A, Rudolf I, Hubálek Z, Földvári G, Plantard O, Vayssier-Taussat M, Bonnet S, Spitalská E, Kazimírová M (2014) *Ixodes ricinus* and Its transmitted pathogens in urban and peri-urban areas in Europe: New Hazards and relevance for public health. *Front Public Health.* 2: 251.
 18. Wilhelmsson P, Pawełczyk O, Jaenson TG, Waldenström J, Olsen B, Forsberg P, Lindgren PE (2021) Three *Babesia* species in *Ixodes ricinus* ticks from migratory birds in Sweden. *Parasit Vectors.* 14(1): 183.
 19. Pettersson JH-O (2013) The Origin of the Genus *Flavivirus* and the Ecology of Tick-Borne Pathogens, PhD Thesis, Acta Universitatis Upsaliensis.
 20. Blanda V, Torina A, La Russa F, D'Agostino R, Randazzo K, Scimeca S, Giudice E, Caracappa S, Cascio A, de la Fuente J (2017) A retrospective study of the characterization of *Rickettsia* species in ticks collected from humans. *Ticks Tick Borne Dis.* 8(4): 610–614.
 21. Cutler SJ, Ruzic-Sabljic E, Potkonjak A (2017) Emerging borreliae—Expanding beyond Lyme borreliosis. *Mol Cell Probes.* 31: 22–27.
 22. Mansfield KL, Cook C, Ellis RJ, Bell-Sakyi L, Johnson N, Alberdi P, de la Fuente J, Fooks AR (2017) Tick-borne pathogens induce differential expression of genes promoting cell survival and host resistance in *Ixodes ricinus* cells. *Parasit Vectors.* 10(1): 81.
 23. Reis C, Cote M, Le Rhun D, Lecuelle B, Levin ML, Vayssier-Taussat M, Bonnet SI (2011) Vector competence of the tick *Ixodes ricinus* for transmission of *Bartonella birtlesii*. *PLoS Negl Trop Dis.* 5(5): e1186.
 24. Pajoro M, Pistone D, Varotto Boccazzi I, Mereghetti V, Bandi C, Fabbi M, Scattorin F, Sasserà D, Montagna M (2018) Molecular screening for bacterial path-

- ogens in ticks (*Ixodes ricinus*) collected on migratory birds captured in northern Italy. *Folia Parasitol.* 65: 2018.008.
25. Laaksonen M, Klemola T, Feuth E, Sormunen JJ, Puisto A, Mäkelä S, Penttinen R, Ruohomäki K, Hänninen J, Sääksjärvi IE, Vuorinen I, Sprong H, Hytönen J, Vesterinen EJ (2018) Tick-borne pathogens in Finland: comparison of *Ixodes ricinus* and *I. persulcatus* in sympatric and parapatric areas. *Parasit Vectors.* 11(1): 556.
 26. Song X, Zhong Z, Gao L, Weiss BL, Wang J (2022) Metabolic interactions between disease-transmitting vectors and their microbiota. *Trends Parasitol.* 38(8): 697–708.
 27. Fukuda K, Ogawa M, Taniguchi H, Saito M (2016) Molecular approaches to studying microbial communities: targeting the 16S ribosomal RNA gene. *J UOEH.* 38(3): 223–232.
 28. Mathison BA, Pritt BS (2014) Laboratory identification of arthropod ectoparasites. *Clin Microbiol Rev.* 27(1): 48–67.
 29. Salehi Z, Shams-Ghahfarokhi M, Fattahi A, Ghazanfari M, Yazdanparast SA (2018) A head-to-head comparison of four cryopreservation protocols of dermatophyte species. *Infect Epidemiol Microbiol.* 4(3): 109–114.
 30. Patel RK, Jain M (2012) NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. *PloS One.* 7(2): e30619.
 31. Okonechnikov K, Conesa A, García-Alcalde F (2016) Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics.* 32(2): 292–294.
 32. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson A. JA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods.* 13(7): 581–583.
 33. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41(Database issue): D590–596.
 34. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Annicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciulek T, Krepis J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ull-Hasan S, Van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 37(8): 852–857.
 35. Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics.* 30(9): 1312–1313.
 36. Carpi G, Cagnacci F, Wittekindt NE, Zhao

- F, Qi J, Tomsho LP, Drautz DI, Rizzoli A, Schuster SC (2011) Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. *PLoS One*. 6(10): e25604.
37. Portillo A, Palomar AM, de Toro M, Santibáñez S, Santibáñez P, Oteo JA (2019) Exploring the bacteriome in anthropophilic ticks: To investigate the vectors for diagnosis. *Plos One*. 14(3): e0213384.
 38. Choubdar N, Karimian F, Koosha M, Oshaghi MA (2021) An integrated overview of the bacterial flora composition of *Hyalomma anatolicum*, the main vector of CCHF. *PLoS Negl Trop Dis*. 15(6): e0009480.
 39. Batool M, Blazier JC, Rogovska YV, Wang J, Liu S, Nebogatkin IV, Rogovskyy AS (2021) Metagenomic analysis of individually analyzed ticks from Eastern Europe demonstrates regional and sex-dependent differences in the microbiota of *Ixodes ricinus*. *Ticks Tick Borne Dis*. 12(5): 101768.
 40. Kmet' V, Čaplová Z (2019) An update on the *Ixodes ricinus* microbiome. *J Microbiol Biotechnol Food Sci*. 8(6): 1340–1342.
 41. Sacchi L, Bigliardi E, Corona S, Beninati T, Lo N, Franceschi A (2004) A symbiont of the tick *Ixodes ricinus* invades and consumes mitochondria in a mode similar to that of the parasitic bacterium *Bdellovibrio bacteriovorus*. *Tissue Cell*. 36(1): 43–53.
 42. Lo N, Beninati T, Sasser D, Bouman EA, Santagati S, Gern L, Sambri V, Masuzawa T, Gray JS, Jaenson TG, Bouattour A, Kenny MJ, Guner ES, Kharitonov IG, Bitam I, Bandi C (2006) Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*. *Environ Microbiol*. 8(7): 1280–1287.
 43. Lejal E, Chiquet J, Aubert J, Robin S, Estrada-Peña A, Rue O, Midoux C, Mariadassou M, Bailly X, Cougoul A, Gasqui P, Cosson JF, Chalvet-Monfray K, Vayssier-Taussat M, Pollet T (2021) Temporal patterns in *Ixodes ricinus* microbial communities: an insight into tick-borne microbe interactions. *Microbiome*. 9(1): 153.
 44. Porter SR, Czaplicki G, Mainil J, Guattéo R, Saegerman C (2011) Q Fever: current state of knowledge and perspectives of research of a neglected zoonosis. *Int J Microbiol*. 2011: 248418.
 45. Mostafavi E, Rastad H, Khalili M (2012) Q fever: an emerging public health concern in Iran. *Asian J Epidemiol*. 5(3): 66–74.
 46. Hosseini-Chegeni A, Tavakoli M, Telmadarraiy Z, Faghihi F (2020) Molecular detection of spotted fever group *Rickettsia* (Rickettsiales: Rickettsiaceae) in ticks of Iran. *Arch Razi Inst*. 75(3): 317–325.
 47. Eybpoosh S, Fazlalipour M, Baniasadi V, Pouriayevali MH, Sadeghi F, Ahmadi Vasmehjani A, Karbalaie Niya MH, Hewson R, Salehi-Vaziri M (2019) Epidemiology of West Nile Virus in the Eastern Mediterranean region: A systematic review. *PLoS Negl Trop Dis*. 13(1): e0007081.
 48. Ghaderi E, Salehi Vaziri M, Mostafavi E, Moradi G, Rahmani K, Zeinali M, Shirzadi MR, Erfani H, Afrasiabian SH, Eybpoosh S (2019) Evaluation of Crimean-congo Hemorrhagic Fever (CCHF) Surveillance System in Iran. *Iran J Epidemiol*. 15(3): 300–312.
 49. Duval BD, Elrod MG, Gee JE, Chantratita N, Tandhavanant S, Limmathurotsakul D, Hoffmaster AR (2014) Evaluation of a latex agglutination assay for the identification of *Burkholderia pseudomallei* and *Burkholderia mallei*. *Am J Trop Med Hyg*. 90(6): 1043–1046.
 50. Pal M (2005) Importance of zoonoses in public health. *Indian J Anim Sci*. 75(5): 586–591.
 51. Brinkerhoff RJ, Clark C, Ocasio K, Gauthier DT, Hynes WL (2020) Factors affecting the microbiome of *Ixodes scapularis* and *Amblyomma americanum*. *PloS One*. 15(5): e0232398.

52. Van Treuren W, Ponnusamy L, Brinkerhoff RJ, Gonzalez A, Parobek CM, Juliano JJ, Andreadis TG, Falco RC, Ziegler LB, Hathaway N, Keeler C, Emch M, Bailey JA, Roe RM, Apperson CS, Knight R, Meshnick SR (2015) Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. *Appl Environ Microbiol.* 81(18): 6200–6209.
53. Cronin P, Joyce SA, O'Toole PW, O'Connor EM (2021) Dietary fibre modulates the gut microbiota. *Nutrients.* 13(5): 1655.