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# Characterization of microbiota diversity of engorged ticks collected from dogs in China

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## ABSTRACT

**Background:** Ticks are one of the most common external parasites in dogs, and are associated with the transmission of a number of major zoonoses, which result in serious harm to human health and even death. Also, the increasing number of pet dogs and pet owners in China has caused concern regarding human tick-borne illnesses. Accordingly, studies are needed to gain a complete understanding of the bacterial composition and diversity of the ticks that parasitize dogs.

**Objectives:** To date, there have been relatively few reports on the analysis of the bacterial community structure and diversity in ticks that parasitize dogs. The objective of this study was to investigate the microbial composition and diversity of parasitic ticks of dogs, and assessed the effect of tick sex and geographical region on the bacterial composition in two tick genera collected from dogs in China.

**Methods:** A total of 178 whole ticks were subjected to a 16S ribosomal RNA (rRNA) next generation sequencing analysis. The Illumina MiSeq platform targeting the V3–V4 region of the 16S rRNA gene was used to characterize the bacterial communities of the collected ticks. Sequence analysis and taxonomic assignment were performed using QIIME 2 and the GreenGene database, respectively. After clustering the sequences into taxonomic units, the sequences were quality-filtered and rarefied.

**Results:** After pooling 24 tick samples, we identified a total of 2,081 operational taxonomic units, which were assigned to 23 phyla and 328 genera, revealing a diverse bacterial community profile. The high, moderate and low prevalent taxa include 46, 101, and 182 genera, respectively. Among them, dominant taxa include environmental bacterial genera, such as *Psychrobacter* and *Burkholderia*. Additionally, some known tick-associated endosymbionts were also detected, including *Coxiella*, *Rickettsia*, and *Rickettsiella*. Also, the potentially pathogenic genera *Staphylococcus* and *Pseudomonas* were detected in the tick pools. Moreover, our preliminary study found that the differences in microbial communities are more dependent on the sampling location than tick sex in the tick specimens collected from dogs.

**Conclusions:** The findings of this study support the need for future research on the microbial population present in ticks collected from dogs in China.

**Keywords:** Microbiome; China; tick; microbial community; dog

**Conflict of Interest**

The authors declare no conflicts of interest.

**Author Contributions**

Conceptualization: Hua X, Cui L; Data

curation: Wang S; Formal analysis: Wang S;

Funding acquisition: Cui L; Investigation: Wang

S; Supervision: Cui L; Visualization: Wang

S; Writing - original draft: Wang S; Writing -

review & editing: Cui L.

**INTRODUCTION**

Dogs are the most common household pets in China. Recent reports have revealed that as of 2018, dogs were owned by about 33.9 million people or 46.1 per cent of total pet owners, who owned 50.08 million dogs in China [1]. The increasing number of households with pet dogs in China have caused a variety of pet-related issues, including zoonotic diseases [2]. Also, a recent study has found that pet ownership is significantly associated with increased tick encounters for their owners [3]. Accordingly, among the diseases common to dogs and humans, tick-borne diseases have received extensive attention since ticks are among the most frequently encountered external parasites in dogs, and are one of the most common vectors of a number of major zoonoses [4,5]. As a result, dogs have become sentinels and reservoirs for human tick-borne diseases in recent years [6]. *Haemaphysalis* and *Rhipicephalus* are recognized as the most commonly reported tick genera in China. They are capable of transmitting a broad range of bacterial pathogens, and have been implicated as competent vectors, which are harmful to human and domestic animal health [6-8]. Accordingly, there is a large body of the research work focusing on the bacterial diversity and community composition of these tick genera collected from domestic animals [9,10]. To date, the bacterial community structure and diversity in ticks that parasitized dogs have not been thoroughly studied, and additional studies are needed to gain a better insight into the composition and diversity of the bacterial community in ticks that parasitize dogs. Microbial community of ticks can be influenced by many factors, including geographical region, blood meal source, state of feeding and developmental stages [11]. Among them, shifts in bacterial composition of ticks induced by sex and geographical region could be a key determinant of microbial variation. Current 16S ribosomal RNA (rRNA) gene sequence profiling techniques based on the canonical clustering threshold of 97% identity for operational taxonomic units (OTUs) are not adequate for accurate taxonomic assignment to resolve species-level phylogenetic relationships when just a few hypervariable regions of the gene are amplified [12]. Accordingly, the 16S rRNA gene amplicon sequence data using the V3-V4 region should be explored at the genus level only [13]. In this study, we used the Illumina MiSeq platform for 16S rRNA amplicon analysis, built small fragment libraries, and performed sequencing using the paired-end methods to characterize the microbial composition of the ticks in dogs. In this preliminary study, we evaluated the effect of tick sex and geographical region on the bacterial composition of the four tick species collected from dogs in China.

**MATERIALS AND METHODS****Collection of ticks and sample preparation**

A total of 178 feeding ticks collected from dogs in a previous study by Wang et al. [1] were used for this study. After collection, the ticks were morphologically identified and grouped according to their sampling sites and sex (Table 1). Four tick species frequently found in China were detected, namely *Haemaphysalis longicornis*, *Haemaphysalis flava*, *Rhipicephalus sanguineus*, and *Rhipicephalus turanicus* [11,14]. Subsequently, the ticks were kept at -80°C in a plastic tube for subsequent studies.

**DNA extraction and purification from tick samples**

For DNA extraction, whole ticks were sterilized with 75% ethanol for 5 min followed by washing in sterile deionized water. DNA extraction was performed using the QIAamp DNA Micro Kit following the manufacturer's instructions. The Picogreen quantification reagent

**Table 1.** Sample of engorged ticks collected from dogs used in this study

Library number	Sampling sites	Sex	Number of ticks pooled	Collection date	SRA Accession number
1a	Shanghai, Police dog station	Male	7	201804	SRR10917484
2a	Shanghai, Pudong, A	Male	7	201803	SRR10917484
3a	Shanghai, Pudong, B	Male	13	201804	SRR10917483
4a	Shanghai, Pudong, C	Male	8	201806	SRR10917483
5a	Shanghai, Minhang, A	Male	12	201803	SRR10917482
6a	Shanghai, Minhang, B	Male	7	201805	SRR10917482
7a	Shanghai, Minhang, C	Male	6	201806	SRR10917481
8a	Shanghai, Minhang, D	Male	8	201807	SRR10917481
9a	Liaoning, Shenyang	Male	7	201808	SRR10917490
10a	Henan, Zhumadian	Male	22	201808	SRR10917489
11a	Jiangxi, Nanchang	Male	6	201808	SRR10917488
12a	Shanghai, Xuhui	Male	17	2017	SRR10917480
1b	Shanghai, Police dog station	Female	3	201804	SRR10917484
2b	Shanghai, Pudong, A	Female	4	201803	SRR10917484
3b	Shanghai, Pudong, B	Female	3	201804	SRR10917483
4b	Shanghai, Pudong, C	Female	5	201806	SRR10917483
5b	Shanghai, Minhang, A	Female	5	201803	SRR10917482
6b	Shanghai, Minhang, B	Female	4	201805	SRR10917482
7b	Shanghai, Minhang, C	Female	3	201806	SRR10917481
8b	Shanghai, Minhang, D	Female	5	201807	SRR10917481
9b	Liaoning, Shenyang	Female	4	201808	SRR10917490
10b	Henan, Zhumadian	Female	10	201808	SRR10917489
11b	Jiangxi, Nanchang	Female	4	201808	SRR10917488
12b	Shanghai, Xuhui	Female	8	2017	SRR10917480

(Invitrogen, USA) and the FLx800 fluorescence microplate reader (BioTek, USA) were used to quantify the total DNA after DNA extraction. Then, DNA was purified using AMPure XP beads (Beckman Coulter, USA).

### Sequencing of the V3–V4 region of the 16S rRNA gene and data analysis

The 16S rRNA gene sequences were amplified by polymerase chain reaction using a set of primers (forward primer 5'-GCACCTAAYTGGGYDTAAAGNG-3' and reverse primer 5'-TACNVGGGTATCTAATCC-3'). The resulting libraries were sequenced using the TruSeq Nano DNA LT library preparation kit (Illumina Inc., USA) on an Illumina Miseq platform (2 × 300 bp paired-end reads) following the manufacturer's instructions. Then, the library quality was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies Inc., USA). The V3–V4 amplified region (200–450 bp) was demultiplexed and quality controlled using QIIME 2 [15]. In short, sequences were trimmed according to the following criteria: 1) Phred quality score ≥ 20; 2) sequence length > 200 bp; and 3) no ambiguous sequences. Next, the reads were clustered into OTUs performed using an identity threshold of 97% and the GreenGene database [16]. After clustering, the sequences were assigned taxonomy with the UCLUST clustering algorithm and following the open-reference OTUs picking protocol in QIIME 2 [17]. The OTUs (chimeric and spurious) with a relative abundance of < 0.001% of the total read counts were removed. All resulting sequences are available through the NCBI BioProject (ID PRJNA599265). For data analyses, the Chao1 index for richness, Shannon and Simpson diversity indices were estimated using the QIIME 2 software and visualized by the R software v 3.1.2 [18]. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to determine the microbial differences between sexes in the collected ticks. In addition, analyses of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed to compare the microbial composition among the groups using weighted and unweighted UniFrac metrics. Additionally, principal coordinate analysis (PCoA) plot was used for data visualization. The results were considered statistically significant for *p* value ≤ 0.05.

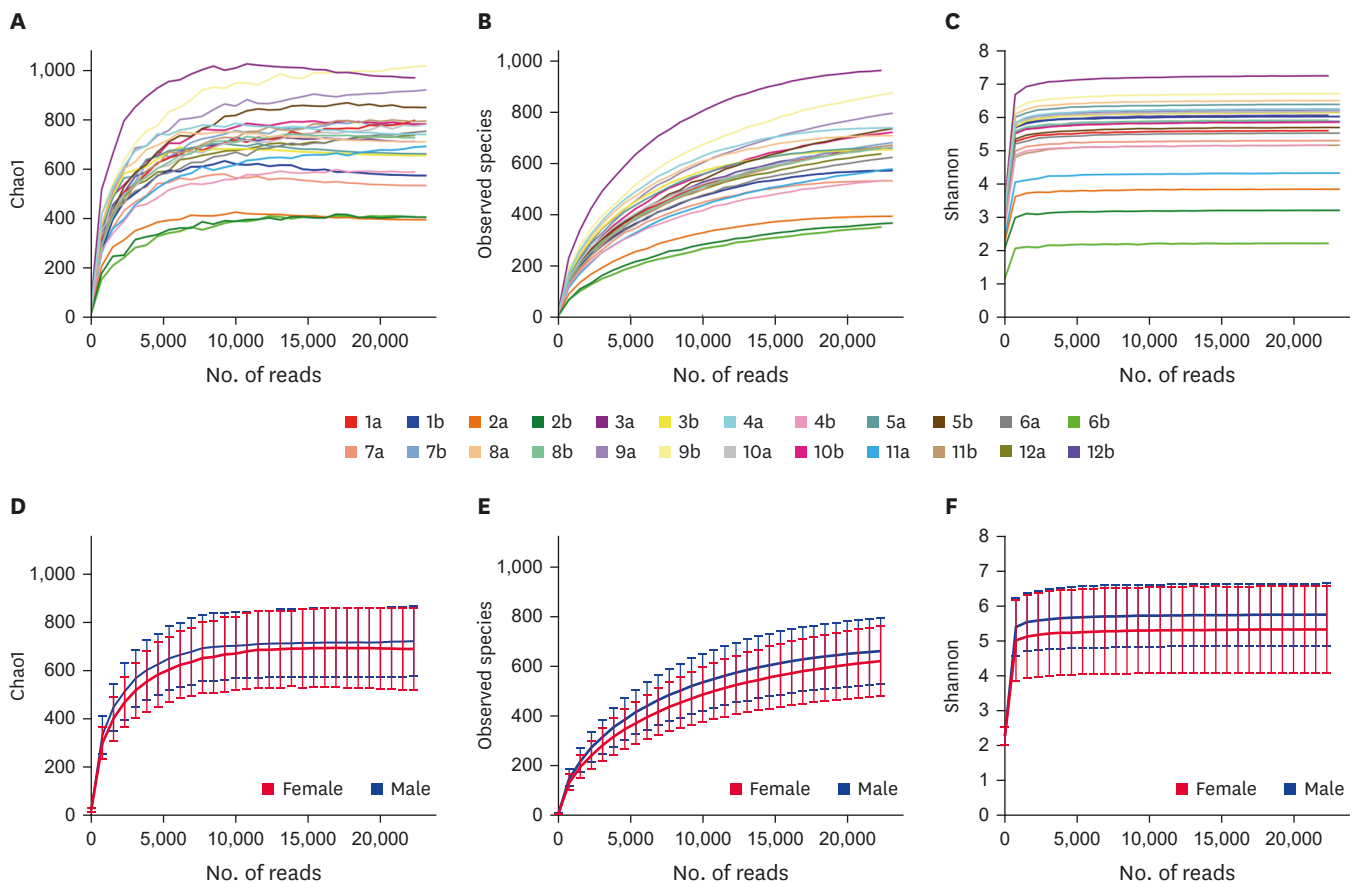
## RESULTS

### The 16S rRNA gene V3–V4 amplicon sequencing results

For 24 pools of tick DNA samples ( $n = 178$ ), sequencing of the amplicon of the V3–V4 region of the bacterial 16S gene on the MiSeq platform produced 1,004,202 reads. The initial quality filtering step in the QIIME 2 workflow yielded 25,909–85,505 reads for downstream analysis. To overcome the possible effects of the unequal sequencing output across samples, the reads were rarefied to 25,000 reads for use in OTUs picking procedure and taxonomic assignment. The observed rarefaction curves and Chao1 rarefaction curves reached a plateau for all samples (**Fig. 1**). These results show that the sequencing depth was sufficient to estimate 99% of the bacterial diversity and species richness in all samples.

### Taxonomic analysis of 16S rRNA gene sequencing data

Using this approach, a total of 2,081 OTUs were identified, which were assigned to 23 phyla and 328 genera. The majority of the bacterial sequences were assigned to Proteobacteria (1,446 OTUs), followed by Firmicutes (303 OTUs), Bacteroidetes (131 OTUs), Actinobacteria (103 OTUs), Acidobacteria (34 OTUs) and Cyanobacteria (14 OTUs). The lowest number of observed OTUs was 417 in sample 6b, while the highest was 1,014 in sample 3a (**Table 2**). There was 1 unclassified OTU at the genus level. The bacterial calculated richness varied from 395 to 1,015.67 per sample (**Table 2**).



**Fig. 1.** Rarefaction curves of bacterial microbiome demonstrating the Chao1 index (A, D), Observed species (B, E), and Shannon index (C, F) according to each tick sample (upper panel) and the tick sex (lower panel). Data from sequenced reads were rarefied to 25,000 sequences. Error bars represent SEs. F, female; M, male.

**Table 2.** Microbial diversity parameters and relative abundance of major bacteria phyla according to the pooled libraries

Library	Chao1	Total OTUs	No. of reads	Relative abundance (%)						
				Proteobacteria	Firmicutes	Bacteroidetes	Actinobacteria	Acidobacteria	Cyanobacteria	Others
1a	803.57	720	23,040	86.0	3.5	8.2	1.1	0.6	0.4	0.2
1b	573.09	618	23,107	84.3	3.4	8.4	2.8	0.5	0.3	0.3
2a	395	449	23,113	82.2	14.7	1.3	1.7	0.1	0.1	< 0.1
2b	405.68	425	23,086	93.2	5.9	0.2	0.7	0.0	0.0	< 0.1
3a	966.17	1,014	23,042	69.6	16.3	4.3	2.6	0.5	6.0	0.7
3b	654	712	23,082	94.0	1.9	1.8	1.3	0.8	0.1	0.1
4a	739	787	23,092	92.8	2.9	2.0	1.4	0.6	0.1	0.2
4b	585.55	592	23,044	81.5	2.0	13.5	1.4	0.6	0.6	0.4
5a	661	719	23,076	85.0	7.6	4.6	1.3	1.1	0.1	0.3
5b	847.92	793	23,073	93.3	1.4	3.1	1.7	0.4	0.1	< 0.1
6a	751.85	680	23,053	85.0	2.1	10.9	0.8	0.5	0.3	0.4
6b	407.54	417	23,030	96.8	0.6	1.6	0.7	0.1	0.0	0.2
7a	533	590	23,129	83.9	1.9	12.1	1.1	0.4	0.4	0.2
7b	778.97	738	23,110	85.9	2.7	7.9	2.4	0.6	0.2	0.3
8a	709	763	23,055	86.7	5.6	3.7	2.4	1.0	0.2	0.4
8b	721.75	719	23,000	91.4	2.2	4.1	1.7	0.5	0.1	< 0.1
9a	918.5	853	23,066	87.2	3.8	6.5	1.3	0.8	0.1	0.3
9b	1015.67	913	23,063	84.9	3.5	4.4	4.1	1.6	0.2	1.3
10a	778.99	721	23,126	84.7	7.3	5.6	1.2	0.7	0.1	0.4
10b	781.32	776	23,074	71.9	21.6	3.5	2.3	0.5	0.1	0.1
11a	690.42	833	23,088	58.3	39.4	0.8	1.4	0.1	0.0	< 0.1
11b	792.86	726	23,097	55.7	39.0	2.8	2.0	0.2	0.1	0.2
12a	738.81	698	23,049	89.2	2.4	5.9	1.4	0.8	0.2	0.1
12b	706.46	722	23,036	86.0	2.5	5.1	4.6	0.8	0.8	0.2

OTU, operational taxonomic unit.

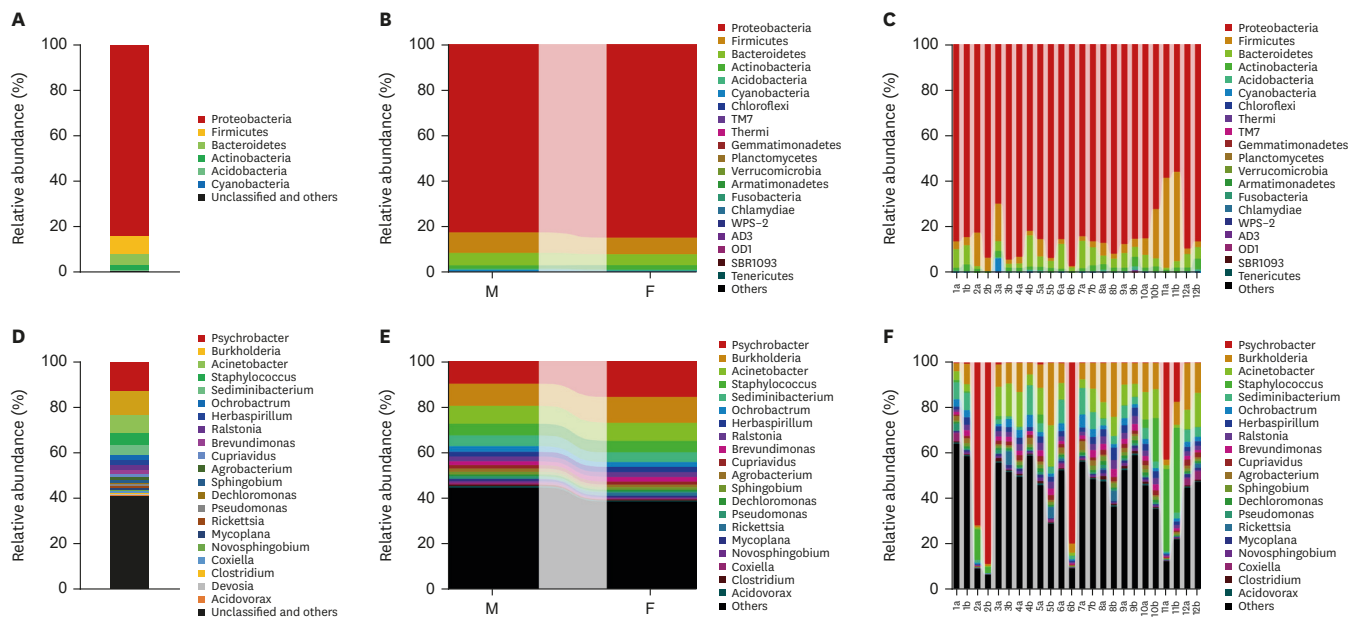
At the phylum level, Proteobacteria was present in greatest abundance at 83.7% (55.7%–96.8%), followed by Firmicutes at 8.1% (0.6%–39.4%), Bacteroidetes at 5.1% (0.2%–10.9%), Actinobacteria at 1.8% (0.7%–4.6%), Acidobacteria at 0.6% (0.1%–1.6%) and Cyanobacteria at 0.4% (0.1%–6.0%), which were the prevalent phyla in all samples. On the other hand, an additional 17 phyla, including Chloroflexi, Thermi and TM7, were rarely detected and were low to moderately prevalent in the samples (**Fig. 2**).

At the genus level, *Psychrobacter* belonging to the Proteobacteria phylum was the most abundant, accounting for 12.8% (0.1%–89%), followed by *Burkholderia* 10.5% (0.8%–27.6%), *Acinetobacter* 8% (0.5%–18.3%), *Staphylococcus* 5% (0.1%–37.5%), *Sediminibacterium* 4.5% (0.2%–13%), *Ochrobactrum* 2.3% (0.1%–5.7%), *Herbaspirillum* 2.2% (0.1%–5.5%), *Ralstonia* 2.1% (0.0%–3.8%), and *Brevundimonas* 1.8% (0.1%–3.5%). Other genera with abundance above 1% included *Cupriavidus* (1.4%), *Agrobacterium* (1.3%), *Sphingobium* (1.1%) while the remainder of the detected bacterial genera had abundance of less than 1% (**Fig. 2**). Unclassified genera and other genera with low abundance are listed in **Supplementary Table 1**.

Among 46 high prevalent genera, *Acinetobacter* as well as unclassified *Comamonadaceae* and *Burkholderia* were typically the most abundant taxa for all samples. Other genera, such as unclassified *Sphingomonadaceae*, unclassified *Sediminibacterium*, and unclassified *Sediminibacterium* as well as unclassified *Moraxellaceae*, *Ochrobactrum*, *Herbaspirillum*, *Ralstonia*, and *Brevundimonas* were always present, but at relatively low abundance from the tick pools. *Staphylococcus* and *Pseudomonas*, the potentially pathogenic genera also were detected and were the 5th and 21st most abundant taxa, respectively, among all samples.

Among 101 moderately prevalent genera, we found Endosymbionts and pathogenic bacteria that are of public health significance. Among the possible endosymbionts in ticks, *Coxiella*





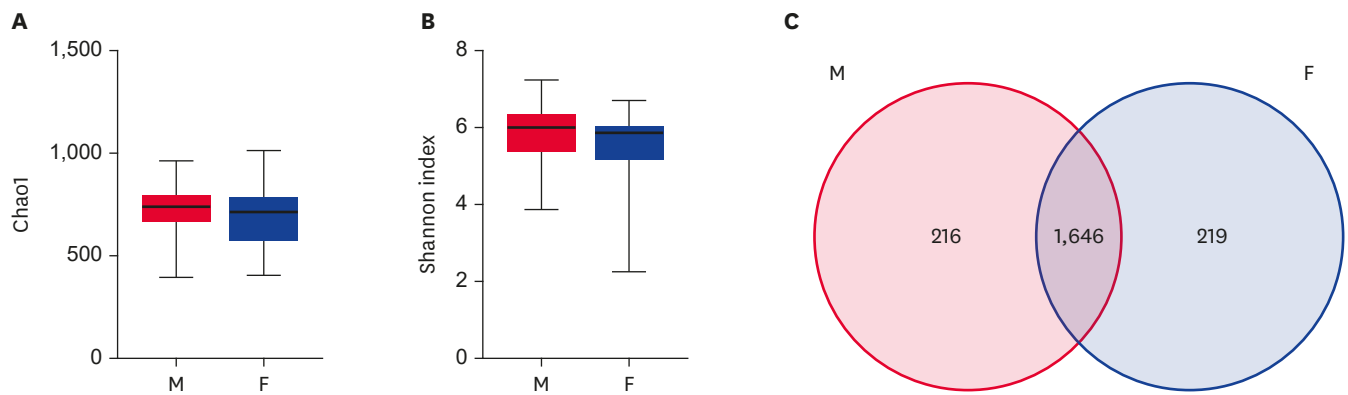
**Fig. 2.** Relative abundance of bacteria at the phylum level (upper panel) and genus level (lower panel). (A, D) Classification based on the whole library. (B, E) Classification based on the sex. (C, F) Classification based on each library. Data are presented in proportion (%) to all sequences classified at this taxonomy level. “Others” denotes the total relative abundance of phyla except for the stated phyla.

accounted for 0.6% of the total reads, and was detected in 20 out of 24 samples, ranging from 0 to 3.7% in the 20 sample. Also, *Rickettsiella* was detected at very low abundance (0.1%) in 5 out of 24 samples. *Rickettsia*, a potential pathogen of human, was also found in 18 out of 24 samples, and accounted for 0.8% of the total reads, ranging from 0 to 6% in the 18 sample. However, other medically important tick bacteria genera that cause serious tick-borne diseases including *Borrelia*, *Anaplasma*, *Ehrlichia*, *Bartonella*, *Wolbachia*, and *Francisella*, were not detected in this study.

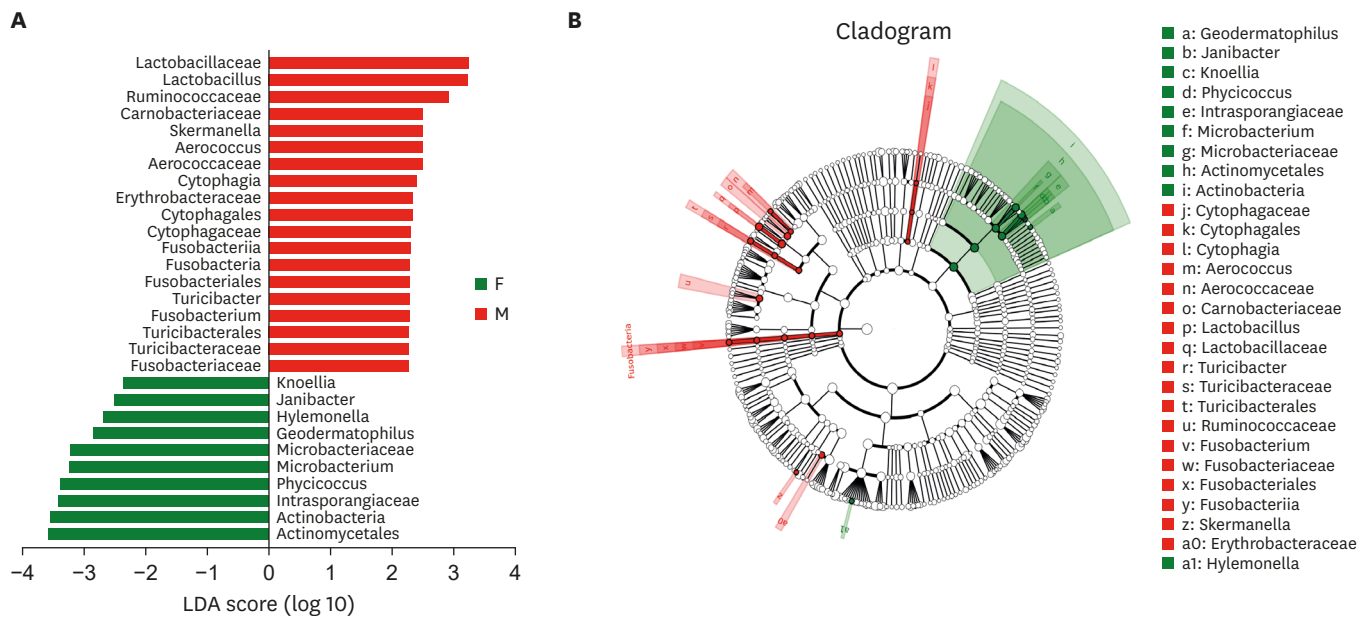
Among 182 low prevalent genera, we also found certain pathogenic genera, such as *Enterobacter* (11/24), *Klebsiella* (3/24), *Lactococcus* (5/24), which have species previously detected in human clinical specimens. The lower prevalence and abundance of pathogenic bacteria suggest that they are potential opportunistic pathogens acquired from the surrounding environment [19]. Endosymbiotic bacteria inhabit many tick species and most frequently predominant bacterial community.

### Comparison of the microbiota between male and female engorged collected ticks

Alpha diversity analysis revealed that male ticks exhibited greater microbial diversity than female ticks. However, there were no significant differences in bacterial richness ( $p = 0.7125$ ) and diversity ( $p = 0.3546$ ) between male and female group. A total of 1,646 OTUs were shared by both sexes, with 216 and 219 OTUs unique to female and male ticks, respectively (Fig. 3). In further assessments of differences in the bacterial community composition, weighted and unweighted UniFrac analysis revealed no difference with regard to sex, as measured by ANOSIM and PERMANOVA. The microbiome composition difference between the groups was determined by LefSe analysis. In total, 29 bacterial taxa were identified by LDA, score > 2.0 and  $p$  value less than 0.05 (Fig. 4). *Lactobacillaceae* and *Lactobacillus* were highly abundant in males, whereas six bacterial taxa, including Actinomycetales, Actinobacteria, Intrasporangiaceae, *Phycoccus*, *Microbacterium*, and Microbacteriaceae were significantly abundant in females (selected by LDA score > 3.0).



**Fig. 3.** Alpha diversity measures and operational taxonomic units comparison diagram between male and female ticks. F, female; M, male.



**Fig. 4.** (A) Histogram of the LDA scores (> 2) computed for bacterial taxa differentially abundant between male and female. The size of each node represents the LDA score. (B) Bacterial taxa that were differentially abundant in the group visualized using a cladogram. The size of each node represents their relative abundance. Significantly discriminant taxon nodes are colored and branch areas are shaded according to the highest-ranked variety for that taxon. F, female; M, male; LDA, linear discriminant analysis.

### Comparison of engorged ticks collected from different sampling locations

We analyzed the microbial composition of engorged ticks collected from different sampling locations by using the weighted and unweighted Unifrac distances with statistical analysis. Both weighted and unweighted UniFrac analysis and PCoA plot showed that a pair of samples with different sex from the same collection site tended to be segregated together. In addition, there was a clear separation of the 12 different sampling sites based on the statistical analysis. Significant difference was detected among the bacterial composition of the tick pools collected at different sampling sites (ANOSIM  $R = 0.5107$ ,  $p = 0.001$ ; PERMANOVA  $R^2 = 0.59844$ ,  $p = 0.001$ ), visualized by the PCoA plot, with the first and second principal coordinates explaining 21.84 and 7.41% of the variation for unweighted UniFrac measures (Fig. 5).

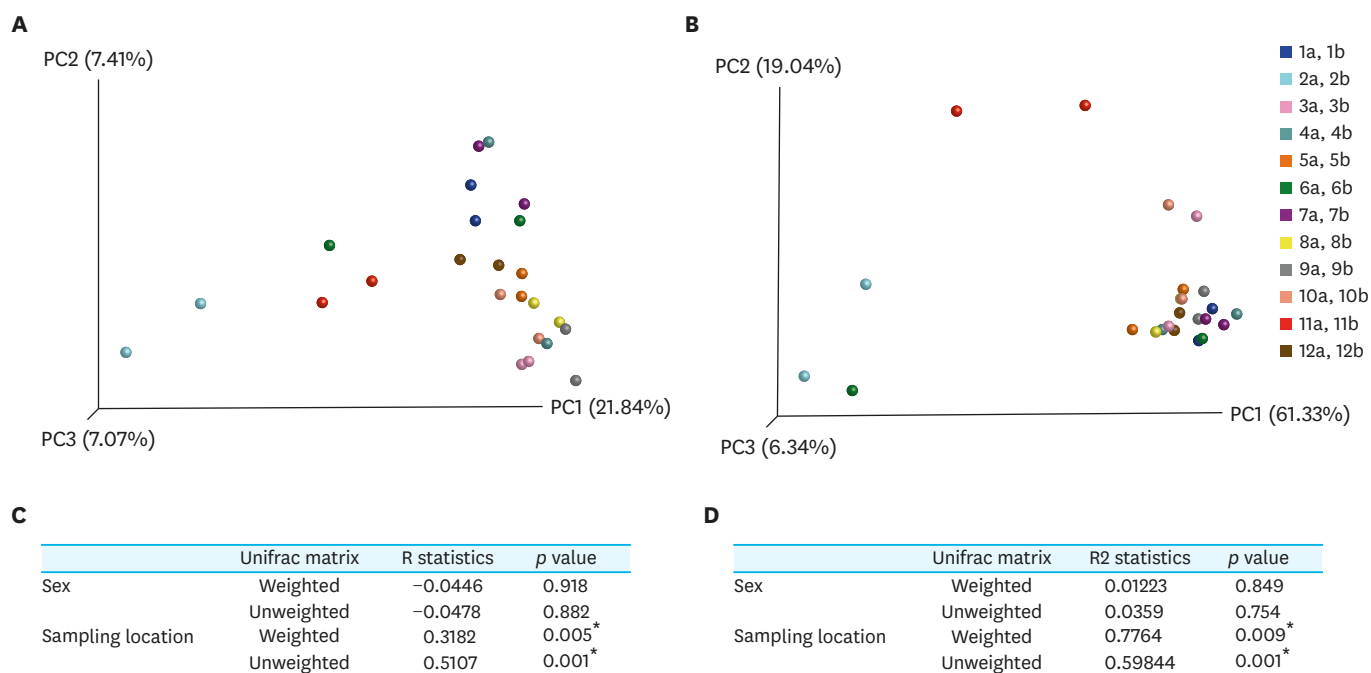


Fig. 5. First 2 principal coordinate analysis plot using unweighted (A) and weighted (B) UniFrac distance of the bacterial composition based on the different tick sampling sites. (C, D) show the results of the analysis of similarity and the permutational analysis of variance, respectively, based on the sex or sampling location.

## DISCUSSION

In this study, we investigated the bacterial community in ticks collected from pet dogs in China. The bacteriome of the collected ticks was analyzed by sequencing the V3–V4 region of the 16S rRNA gene using the Illumina MiSeq platform. Different from most reported studies have focused on the microbial composition of certain tick species from different hosts, our study characterized the microbiome of non-specific tick species feeding on domesticated dogs. Zhang et al. [10] reported that the source of the blood meals has an important effect on the microbial composition and diversity of ticks, which is likely determined by the different host species and physiological status of the host [11]. We obtained a total of 954,240 high quality reads after removal of low quality and chimeric reads. In total, 328 bacterial taxa were identified to the genus level in this study. Although these results are not directly comparable, microbiome studies performed using these four tick species from different animals and environment, revealed the variation in the number of bacterial genera. Previous studies using metagenomic sequencing have found that *Haemaphysalis* and *Rhipicephalus* spp. harbor between 100 to 200 genera [7]. The relatively high number of bacterial genera found in our study may be due to the environmental contaminants originated from soil or host skin despite our effort in washing the sample [10]. Another possible reason is the variations in the sequencing platform or analysis procedures used [7]. Distinctive bacterial compositions pooled by four different tick species feeding on dogs could be another potential explanation. Consistent with earlier studies, we found that Proteobacteria was the most abundant phylum in the detected tick species [10,11,20,21], with *Psychrobacter* showing the highest abundance. However, previous studies have reported that the most abundant genus present in other tick samples was *Coxiella* (40%–50%) [7,10,11]. Also, Vila *et al.* found that *Coxiella* was the predominant genus in engorged ticks [22,23]. Previous research has suggested that the difference in bacterial richness and diversity



between males and females is due to the different proportions of the *Coxiella* genus in the tick pools [10,11]. However, according to the alpha and beta diversity analyses performed in this study, there were no statistically significant differences in microbiome composition between male and female ticks feeding on dogs. Also, according to our LEfSe analysis with P-value of less than 0.05 and LDA scores greater than 3, Actinomycetales, Actinobacteria, Intrasporangiaceae, *Phycococcus*, *Microbacterium*, Microbacteriaceae, Lactobacillaceae, and *Lactobacillus* were significantly abundant in different sexes with *Coxiella* not showing any significant difference. In our study, the *Coxiella* genus was identified in 20 out of 24 tick pools (83.3%) with a 0.6% relative abundance, which indicated a relatively lower prevalence and abundance than in previous studies [10,11,23]. Multiple factors, such as competition among symbionts, increased virulence, and bottlenecks experienced by symbionts during vertical transmission have been proposed to explain the low proportion of endosymbionts [24]. *Coxiella* is known to encode enzymes involved in the synthesis of vitamin and amino acid which are not acquirable in sufficient quantities from blood meals [25,26]. However, the presence of *Coxiella* endosymbionts does not seem to be absolutely necessary for the tick survival [7]. Accordingly, it is possible that some tick pools may not harbor any *Coxiella* endosymbionts at all or not all individuals of the same tick species carry the endosymbionts at similar proportion as shown in the present study [7]. Recent studies have shown that the relative abundance of *Coxiella* decreased in fed ticks with a gradual increase in microorganism obtained from hosts [10,23]. We have made the assumption that the difference in research findings, to a certain extent may be due to the blood-derived microorganisms acquired from the multiple feeding of the collected ticks from different developmental stages, which resulted in the reduction of the relative amount of *Coxiella* genus [23]. Besides *Coxiella*, *Rickettsia* has been considered to be a predominant bacterial endosymbiont involved in sex determination and biosynthesis of folic acid in *Ixodes* spp. [23,27,28]. Although *Rickettsia* was detected in 18 samples in this study, more than 1,000 bacterial reads were observed in only 3 female samples (3b, 5b, 8b). Our results also showed a similar prevalence and abundance of the Rickettsiaceae family in the tick pools collected from dogs in Palestine [9]. Further research is needed to study the low abundance of the *Rickettsia* genus in engorged tick samples from dogs.

It has been reported that vertebrate host skin microflora and microorganisms in the vertebrate host's blood are brought about by microbial interactions within the tick [29]. We assume that the engorged tick samples contain some bacteria derived from the skin of the dogs on which the ticks were feeding. Coincidentally, the most abundant phylum inhabiting the dog skin is either Proteobacteria or Firmicutes or a combination of both, and both were the most frequently detected phyla in our tick pools [30]. Notably, *Psychrobacter* and *Staphylococcus* have been frequently detected with relatively high abundance in the skin of dogs, especially in dogs with atopic dermatitis [31,32]. *Psychrobacter* is a genus of gram-negative bacteria, which have been associated with food spoilage. It is also a frequently identified genus in the marine environment and aquatic animals as well as in food products [33]. In this study, *Psychrobacter* was present in all samples, with abundance ranging from 0.1 to 89.0% of sequence reads and accounted for 12.8% of average sequences. Although *Psychrobacter* was detected in all samples, it was found to be highly abundant in only 5 tick samples. It is noteworthy that sample 2a, 2b, and 6b exhibited the lowest species richness and high abundance of *Psychrobacter* among all the samples. Except for samples 2a, 2b, and 6b with abundance of > 70%, our findings are similar to those of recent studies showing that *Psychrobacter* constituted < 2% of relative abundance in other tick species [19]. In a recent study, the species of the genus *Psychrobacter* are regarded as medically relevant opportunistic

pathogens and were found to be significantly associated with cutaneous malodour in dogs in the United States [33,34]. *Staphylococcus* are known as common commensals on the dog skin and the high prevalence of *Staphylococcus* in ticks is probably associated with tick survival and reproduction [19,35]. We found that *Staphylococcus* was highly abundant in two samples, namely 11a and 11b. The high abundance of *Staphylococcus* detected in our tick samples could be due to contamination from the host after blood feeding [10]. As *Staphylococcus* is suspected to infect the host reversely after tick contacts, whole genome sequencing and epidemiological study of tick-borne *Staphylococcus* are necessary for efficient animal health surveillance [21]. Besides *Psychrobacter* and *Staphylococcus*, other canine skin bacterial genera including *Clostridium*, *Trichococcus*, *Brachy bacterium*, *Kocuria*, *Turicibacter*, *Dietzia*, *Facklamia*, *Leucobacter*, *Actinomyces*, *Chryseobacterium*, and *Prevotella* were detected, but showed relatively low abundance and prevalence in our study [31]. According to a recent review, the findings of skin and environmental bacteria in ticks may be the result of inadequate sterilization, or that their microbiome may be harbored by ticks during feeding [29].

In addition to pathogenic and endosymbiotic bacteria, environmental and commensal bacteria which can be found in plant and soil were also detected, including *Acinetobacter*, *Corynebacterium*, *Solibacillus*, *Brevundimonas*, *Microbacterium*, *Devosia*, *Sphingomonas*, *Stenotrophomonas*, *Micrococcus*, *Agrobacterium*, *Arthrobacter*, *Flavobacterium*, *Bradyrhizobium*, *Mesorhizobium*, and *Methylobacterium*. They were detected at moderate to high prevalence in our samples and were considered to be innocuous bacteria [36]. According to our data, more than half of the identified genera were found to be present at low prevalence in the tick pools. The low prevalence of 181 genera among tick pools in this study suggests that they might be opportunistic pathogens rather than obligate endosymbionts. Some mammalian intestinal microbiome genera, such as *Ruminococcus*, *Megamonas*, *Lachnospira*, and *Roseburia* and the reported lung microbiota of healthy dogs including *Curvibacter*, *Flavobacteriaceae*, and *Limnhabitans* are examples of possible opportunistic pathogens under stressful conditions [36,37]. An unclassified Comamonadaceae, reported as the indicator genus of mosquitoes fed on host blood meal was found to be the second most abundant taxa in our study [38]. In addition to an unclassified Comamonadaceae, our study detected a substantial proportion of unclassified taxa identified to the genus level (130/328) and 1 unclassified OTUs, which may indicate the presence of yet another uncharacterized novel genus. Zhang et al. [10] reported that the unclassified taxa were present mainly in the eggs and freshly hatched larvae that had no exposure to the external environment [11]. Consequently, more research is needed for the classification and functional verification of the unclassified taxa. Among the genera found in our study, *Sporomusa*, *Flavisolibacter*, *Aminobacter*, *Geosporobacter thermotalea*, and *Xanthobacter* have not been previously isolated from the tick species. Our results can be helpful for future studies of bacteria associated with ticks, and their potential implications for dog health. Some studies have reported that blood feeding is responsible for temporary changes in the microbial compositions. Additionally, the fed ticks showed higher microbial diversity than unfed ticks when the effects of sex and the sampling locations were controlled [10,39]. In our study, the PCoA plot generated with the weighted and unweighted UniFrac metrics, depicting bacterial community patterns revealed that the samples tended to cluster by collecting locations, but not by tick sex. According to our statistical analysis, the samples from the same collection site showed statistical significance with a relatively high dissimilarity (ANOSIM  $R = 0.5107$ , PERMANOVA  $R^2 = 0.59844$ ), whereas the samples from the same tick sex showed no statistically significant difference between groups. Despite using different tick species for this study, our preliminary investigation of the effects of sex and geographical origins revealed that the microbiome composition of engorged ticks was more influenced by

sampling locations than sex. Further research with a larger sample size is required to better understand the interplay of the factors affecting tick microbiome from dogs, including dog species, sampling locations, sex, *etc.*

In summary, our study has characterized the tick microbiome and provides the first baseline data of the microbiome of different engorged tick species collected from dogs in China. The microbiome detected in this study contains a wide range of pathogenic and non-pathogenic microbes for dogs and humans, which were possibly acquired from the environment and host. In particular, our study of four tick species microbiota reveals that the genera *Psychrobacter*, unclassified Comamonadaceae, *Burkholderia*, *Acinetobacter*, and *Staphylococcus* are highly prevalent in ticks. Since our study detected no presence of enzootic pathogens, such as *Ehrlichia*, *Borrelia* and *Anaplasma* in the tick pools, the transmission risk of important tick-borne illnesses in the collecting location is possibly low. In addition, our preliminary investigation of the effects of sex and geographical origins suggests that despite using four different tick species, the sampling site seems to overwhelm the sex component in the tick microbiota.

The results presented in this paper support conducting future research on the microbial population present in ticks collected from dogs in China. Further research including whole genome DNA/RNA sequencing should focus on identifying and differentiating the tick microbiome at the species level. This study has a number of limitations. One of the limitations is that it focused on whole tick bacteriome at the genus level, which prevented us from differentiating between internal bacteria and exoskeleton bacteria of collected ticks. Therefore, a species-level based microbiota study is needed to distinguish between the two groups of bacteria. Another limitation of our study is the small sample size, which prevented us from establishing the geographical presence or absence of some tick-associated pathogens. To ensure biologically meaningful results from diversity study, taking a larger sample size is necessary for validation.

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## SUPPLEMENTARY MATERIAL

### Supplementary Table 1

Highly (100%), moderately (50%–99%) and low prevalent (< 50%) bacteria genera identified in the study

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