RESEARCH ARTICLE
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# Understanding the long-lasting associations among humans, neglected dogs, tick-borne diseases, and core blood bacteria-related pathogenic taxa using next-generation sequencing

S. I. Barraza-Guerrero[1], C. García-De la Peña[2], C. A. Meza-Herrera[3], Q. K. Siller-Rodríguez[2], F. Vaca-Paniagua[4,5,6], C. Díaz-Velásquez[4], A. De la Cruz-Montoya[4], and L. M. Valenzuela-Núñez[2]

1. Department of Health and Hygiene, Antonio Narro Autonomous Agrarian University, Torreón, Coahuila, México. C.P. 27054; 2. Department of Conservation Medicine, Faculty of Biological Sciences, Juarez University of the State of Durango, Gómez Palacio, Durango, México. C.P. 35010; 3. Chapingo Autonomous University, Regional University Unit of Arid Zones, Bermejillo, Durango, México, C.P. 35230; 4. National Health Laboratory: Molecular Diagnosis and Environmental Effect on Chronic-Degenerative Diseases, Faculty of Higher Studies Iztacala, National Autonomous University of Mexico, Tlalnepantla, Estado de México, México. C.P. 54090; 5. National Cancer Institute, Ciudad de México, México, 14080; 6. Department of Biomedicine, Faculty of Higher Studies Iztacala, National Autonomous University of Mexico, Tlalnepantla, Estado de México, México, 54090.

**Corresponding author:** C. A. Meza-Herrera, e-mail: cmeza2020@hotmail.com

**Co-authors:** SIBG: sergiokun.barraza@gmail.com, CGDP: cristina.garcia@ujed.mx, QKSR: qksr@hotmail.com, FVP: felipe.vaca@gmail.com, CDV: cdiazvelasquez@aol.com, ADCM: audelacm@gmail.com, LMVN: luisvn70@hotmail.com

**Received:** 05-11-2023, **Accepted:** 08-02-2024, **Published online:** 29-02-2024

**doi:** www.doi.org/10.14202/IJOH.2024.63-73 **How to cite this article:** Barraza-Guerrero SI, García-De la Peña C, Meza-Herrera CA, Siller-Rodríguez QK, Vaca-Paniagua F, Díaz-Velásquez C, De la Cruz-Montoya A, and Valenzuela-Núñez LM (2024) Understanding the long-lasting associations among humans, neglected dogs, tick-borne diseases, and core blood bacteria-related pathogenic taxa using next-generation sequencing, *Int. J. One Health*, 10(1): 63–73.

## Abstract

**Background and/or Aim:** Dogs are long-lasting companion animals, and ticks are the most common external parasites in dogs. An increase in the population of neglected domestic dogs has increased the risk of contact with ticks, especially in places where tick-borne diseases (TBDs) are endemic. We aimed to characterize the bacterial blood profiles of people who were either exposed (HE) or not exposed (HC) to tick bites using next-generation sequencing (NGS).

**Materials and Methods:** In the present study, the bacteria observed in the blood of people exposed to tick bites were compared with those in the blood of people not exposed to tick bites in Northern Mexico. Human blood samples (n = 12) were analyzed, DNA was extracted, and the V3–V4 region of the 16S ribosomal RNA gene was amplified. In addition, NGS was performed on a MiSeq platform (Illumina), and the data were analyzed through Quantitative Insights into Microbial Ecology.

**Results:** Differences in beta diversity were significant. In HEs, several potentially pathogenic bacterial taxa were found to be the most abundant: *Kocuria* ($\bar{x}$ = 14.59%), *Staphylococcus* ($\bar{x}$ = 3.05%), and *Treponema* ($\bar{x}$ = 2.93%), in addition to *Chlamydia*, *Clostridium*, and *Ehrlichia*, which are considered TBDs.

**Conclusion:** This study identified important differences in the bacterial composition of the HE and HC groups. In addition to *Ehrlichia* (a TBD considered a taxon), other bacterial pathogenic taxa, such as *Chlamydia*, *Clostridium*, *Kocuria*, *Staphylococcus*, and *Treponema*, were also observed in the tick bite-exposed group. Future studies with larger sample sizes should provide an improved understanding of the human blood microbiome profile by providing additional evidence of tick exposure, associated TBDs, and other pathogenic bacterial taxa.

**Keywords:** Blood, *Ehrlichia*, microbiome, tick bites, tick-borne diseases, ticks.

## Introduction

In the past two decades, the incidence of tick-borne diseases (TBDs) caused by bacteria has tripled, and the geographic distribution of ticks has spread widely [1]. Ticks are found within arthropods that feed on human blood, which is considered a serious problem in public health worldwide because of the physical damage caused by adhering to the host and the anaphylactic reactions caused by saliva [2]. Moreover, a wide range of microorganisms, including bacteria, viruses, fungi, protozoa, and helminths, inoculate hosts and cause potentially serious diseases in both domestic and wild animals and humans [3]. These disease-causing organisms are considered neglected or linked to poverty and are currently the main priorities of the World Health Organization along with other international organizations [4]. These disease-causing organisms include viruses (i.e., West Nile fever, Crimean hemorrhagic fever-Congo syndrome, Omsk hemorrhagic fever, and Colorado tick fever), bacteria (i.e., human monocytic ehrlichiosis, human granulocytic ehrlichiosis, Q-fever, rocky mountain spotted fever (RMSF), borreliosis, relapsing fever, and tularemia), fungi

(i.e., dermatophytosis), and protozoa (i.e., theileriosis and babesiosis) [5].

In recent years, new pathogens have appeared, mainly because of genetic changes that help spread ticks and their pathogens. For example, six new bacterial TBDs that have not been previously reported by Rodino *et al*. [6] are considered to have emerged in some areas and reemerged in others due to the expansion of urban sprawl and the availability of hosts. The transmission of these agents simultaneously produces a polymicrobial infection, which not only makes diagnosis difficult but also complicates the clinical picture and prognosis, often resulting in lethal outcomes [1]. Blood is not a sterile medium, and there are many bacterial phylotypes, as well as archaea, viruses, and fungi, which live in humans and have different relationships with our body: commensal, mutualistic, or pathogenic. The concept of a healthy human blood microbiome (HBM) has grown substantially, and comparative analyses of bacterial profiles are becoming increasingly common [7].

Comarca Lagunera, located in northern Mexico, is considered to be an endemic and re-emerging area of RMSF, a highly virulent disease with high mortality caused by *Rickettsia rickettsii* [8]. In fact, in this region, the incidence of RMSF ranges from three cases per 10,000 inhabitants, and the highest incidence occurred in Mexico in 2014 [9]. This health problem was attributed to the presence of street dogs associated with the brown dog tick *Rhipicephalus sanguineus* (sensu lato) [8, 9]. In 2022, the highest fatality rate due to *Rickettsia* in Coahuila was recorded. The Secretary of Health reported 231 cases, 29 of which were confirmed, and 20 of these patients died, representing 69% of the fatalities. The RMSF problem is worsened because 60%–75% of cases are misdiagnosed, and appropriate care is not provided in a timely manner [10].

As previously mentioned, TBD is common in veterinary and medical clinical settings. However, these studies have mainly focused on searching for *R. rickettsii* not only in the region but also in all of Mexico, even though RMSF is a highly important disease. Therefore, it is crucial to define the presence of other bacteria in the blood of people exposed to tick bites because some TBDs can have symptoms, such as those in RMSF and another type of TBD, generating, in turn, misdiagnoses, such as human monocytic ehrlichiosis or human granulocytic ehrlichiosis. Based on these findings, we hypothesized that the bacterial profile and dynamics of people exposed to tick bites are different from those of people who have never been exposed to tick bites. Therefore, in this study, we compared the blood bacteria profiles of people bitten by ticks and those not exposed to ticks in Comarca Lagunera, Mexico.

To our knowledge, this study represents the first of its kind, using the metabarcoding tool to compare microbiomes between people exposed to tick bites vs. people not exposed. This approach will provide valuable information for the scientific community interested in public health intending to analyze and control the risks associated with tick bites.

## Materials and Methods

### Ethical approval and Informed consents

All volunteers provided their consent before samples being taken, and they were informed about the handling of the samples and their anonymity. The study was conducted according to the guidelines of the Declaration of Helsinki (i.e., ethical principles for medical research involving human subjects) [11] with Juarez University of the State of Durango approval reference number UJED-FCB-2018-17.

### Study period and location

The study was conducted from August to October 2019 in northern Mexico, Comarca Lagunera, a region where the municipalities of Torreón, Gómez Palacio, Tlahualilo, Mapimí, and Matamoros converge.

### Groups and samples

Blood was collected from 12 apparently healthy volunteers; seven people were not exposed to a tick bite (HC group), and five people were exposed to a tick bite in the previous year (HE group). Blood was collected by venipuncture, after which the puncture area was previously sanitized with 96% alcohol and deposited in Vacutainer K3EDTA tubes (Vacutainer K3E, BD, USA); 3 mL of blood per person was extracted, and ten drops of blood (50 mg wet weight) from each collected sample were deposited in a (BashingBead™; California, USA) lysis tube of a DNA Zymobiomics Kit (Zymo Research™; California, USA); then, 750 µL of lysing/stabilizing solution (Xpedition™; California, USA) was added. Each tube was processed in a cell disruptor (TerraLyzer™; California, USA) for 30 s [12].

### DNA extraction and visualization

DNA was extracted using the Zymobiomics DNA Miniprep Kit (Zymo Research™) in a ultraviolet laminar flow hood with all sterility protocols. The concentration and quality of the DNA obtained from the samples were measured using a Qubit® 3.0. (Invitrogen, Carlsbad, CA, USA) [12].

### 16S ribosomal RNA (16S rRNA) gene amplicon sequencing

Amplification of the V3–V4 region of the 16S rRNA gene was performed using the following primers [13]: S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-21. Subsequently, the Illumina polymerase chain reaction (PCR) protocol was implemented using 12.5 µL of MyTaq™ Ready Mix 1× (Bioline®, London, UK), 1 µL of each primer (10 nM), 5 µL of DNA (25 ng total) and 5.5 µL of ultrapure $H_2O$; the following cycle was used: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 5 min in a Labnet Multigene™ Gradient PCR thermal cycler (Labnet International, Inc., Global,

Edison, NJ, USA). Amplicons were purified using 0.8% Agentcourt® AMPure® XP beads (Beckman Coulter, Inc., Brea, CA, USA). Then, the amplicons were labeled using a Nextera XT Index Kit™ (Illumina, Inc., San Diego, CA, USA) for the creation of the library, following the Illumina protocol [14], using 25 μL of MyTaq™ Ready Mix 1× (Bioline®), 5 μL of each primer (N7xx and S5xx), 5 μL of DNA, and 10 μL of ultrapure $H_2O$; the following cycle was used: 95°C for 3 min; 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 5 min. Finally, quantification, normalization (i.e., equimolar), and next-generation sequencing (NGS) (MiSeq, Illumina, San Diego, CA, USA) of 2 250 paired-end reads were performed following the Illumina 16S protocol [15].

#### Bioinformatic analysis

Sequence analysis was performed using Quantitative Insights into Microbial Ecology [16]. The assembly was performed using PEAR software (paired-end read merger) [17] with Q30. Chimeras were removed using USEARCH method [18]. Operational taxonomic units (OTUs) were selected using UCLUST method [18] at 97% similarity; taxonomy was assigned using the EzBioCloud database, South Korea [19]. Random rarefaction was performed at a depth of 8400 sequences. The Chao1 estimator richness index and Shannon and Simpson alpha diversity indices were subsequently calculated. Nonparametric t-tests (i.e., false discovery rate correction) were applied to test for differences ($p < 0.05$) between populations for each index. The Bray–Curtis beta diversity was calculated; permutational multivariate analysis of variance (PERMANOVA) was applied to test for significant differences ($p < 0.05$) in the blood microbiome between groups, and the results were visualized using principal coordinate analysis (PCoA) in Emperor [20]. Relative bacterial abundance was obtained at all taxonomic levels. The most abundant phyla are represented in stacked bar graphs generated with R, and the families and genera are visualized in a heatmap constructed with Morpheus (https://software.broadinstitute.org/GENE-E/; accessed 15 September 2021). To establish the bacterial taxa at the phylum, family, and genus levels that contributed the most to the differentiation of the blood microbiome between the two populations, a percentage similarity analysis, similarity percentage (SIMPER) [21], was performed using a Bray–Curtis matrix in PAST 4.04, University of Oslo, Norway. SIMPER partitions the Bray–Curtis dissimilarity for every pair of sample units and calculates the average contribution of each taxon to the difference between the sample units (these contributions are relativized so that the average contributions of all species sum to 1); finally, the statistical significance of these contributions is assessed by permuting the group identities [21]. After SIMPER, some of the taxa that contributed the most to the difference between groups were selected, and Mann–Whitney U tests ($p < 0.05$) were applied to test for significant differences between populations. Finally, linear discriminant analysis effect size (LEfSe) analysis was performed to statistically and biologically determine the key biomarkers that contributed the most to the differences between populations. For these clades, the alpha values were <0.05 for the Kruskal–Wallis factorial test and >4.0 for the logarithmic linear discriminant analysis (LDA) score [22]. This analysis was performed on the website http://huttenhower.sph.harvard.edu/lefse/(accessed September 25, 2022).

## Results

An average of 57,253 assembled sequences was obtained for HCs, whereas 110,009 sequences were obtained for HEs. The average number of quality bacterial sequences was 18,270 for HCs and 47,135 for HEs. The mean OTUs were 1892 and 2774.4 for HCs and HEs, respectively (Table-1). The OTUs obtained in the analysis corresponded to 15 phyla for HCs and 23 for HEs, 29 classes for HCs and 54 for HEs, 51 orders for HCs and 96 for HEs, 90 families for HCs and 178 for HEs, 175 genera for HCs and 397 for HEs, and 187 species for HCs and 420 for HEs.

There was no significant difference in the estimated Chao1 index between the two groups ($t = 0.271$, $p < 0.789$); the mean for HCs was 190.92 ± 49.80 and 204.57 ± 106.23 for HEs. The Shannon diversity index was similar between the groups ($t = 1.67$, $p = 0.128$); the mean for HCs was 4.50 ± 0.44, and that for HEs was 5.13 ± 0.74. Simpson's diversity indices were also similar ($t = 0.629$, $p = 0.565$), with means of 0.91 ± 0.03 and 0.93 ± 0.06. Nevertheless, a significant difference was found in the beta diversity between the two groups according to the Bray–Curtis index (PERMANOVA: pseudo-F = 1.74; $p = 0.002$). Our results indicate that both populations are individually diverse; they are significantly different (Figure 1). Moreover, each community has a different bacterial profile, as demonstrated by the specific segregation between groups in PCoA (Figure 2).

The relative abundance analysis showed that the phylum Firmicutes was the most abundant ($\bar{x}$ = 55.54%) in the HCs, followed by Proteobacteria ($\bar{x}$ = 19.92%), Actinobacteria ($\bar{x}$ = 11.69%), and Bacteroidetes ($\bar{x}$ = 5.95%). Similarly, in the HE, the most abundant phylum was Actinobacteria ($\bar{x}$ = 32.02%), followed by Firmicutes ($\bar{x}$ = 26.17%), Proteobacteria ($\bar{x}$ = 17.04%), Bacteroidetes ($\bar{x}$ = 9.11%), and Spirochaetes ($\bar{x}$ = 3.17%) (Figure-3).

At the family level, the most abundant taxon in the HCs was Bacillaceae ($\bar{x}$ = 29.54), followed by Ruminococcaceae ($\bar{x}$ = 10.99), Christensenellaceae ($\bar{x}$ = 8.89), Micrococcaceae ($\bar{x}$ = 4.20), and Halomonadaceae ($\bar{x}$ = 4.11). In the HE, the most abundant taxon was Micrococcaceae ($\bar{x}$ = 17.41), Bacillaceae ($\bar{x}$ = 6.01), Ruminococcaceae ($\bar{x}$ = 4.03), Ruminococcaceae ($\bar{x}$ = 3.94), and

**Table-1:** 16S rRNA V3-V4 region sequences obtained from blood of volunteers exposed (HE) and not exposed (HC) to tick bites in Comarca Lagunera, north of Mexico.

| Location | Total reads | Assembled | Discarded | QS[1] | QB[2] | OTUs[3] |
|---|---|---|---|---|---|---|
| HC1 | 175,290 | 46,752 | 128,538 | 43,249 | 11,405 | 1327 |
| HC2 | 217,267 | 88,206 | 129,061 | 75,542 | 27,138 | 2816 |
| HC3 | 143,397 | 58,033 | 85,364 | 54,139 | 20,690 | 1973 |
| HC4 | 152,472 | 59,719 | 92,753 | 52,483 | 17,880 | 2355 |
| HC5 | 99,672 | 44,804 | 54,868 | 43,911 | 19,649 | 1506 |
| HC6 | 117,165 | 46,005 | 71,160 | 43,618 | 8417 | 1381 |
| HC7 | 133,492 | 57,252 | 76,240 | 52,910 | 22,708 | 1886 |
| Mean | 148,394 | 57,253 | 91,141 | 52,265 | 18,270 | 1892 |
| HE1 | 114,523 | 69,396 | 45,127 | 66,033 | 10,472 | 1923 |
| HE2 | 106,698 | 66,459 | 40,239 | 62,136 | 14,831 | 2373 |
| HE3 | 115,289 | 73,685 | 41,604 | 71,260 | 40,107 | 1692 |
| HE4 | 601,639 | 272,832 | 328,807 | 265,235 | 145,328 | 5853 |
| HE5 | 133,166 | 67,673 | 65,493 | 61,833 | 24,937 | 2031 |
| Mean | 214,263 | 110,009 | 104,254 | 105,299.4 | 47,135 | 2774.4 |

[1]QS=Quality sequences after chimeras' elimination, [2]QB=Quality bacterial sequences, [3]OTUs=Operational taxonomic units

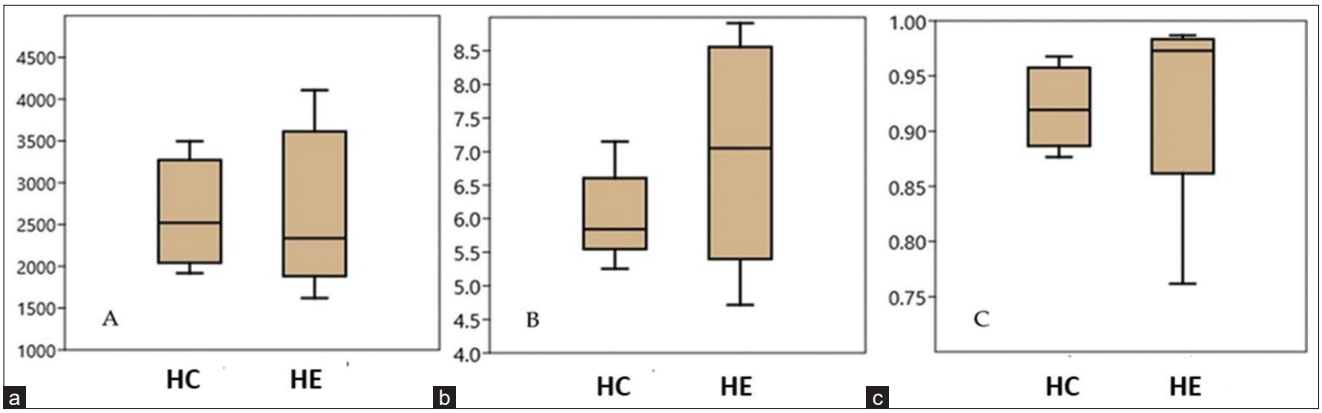


**Figure-1:** Boxplots of (a) Chao1 richness index, (b) Shannon alpha diversity index, and (c) Simpson alpha diversity index for the bacterial microbiota of two groups of volunteers (not exposed = HCs and exposed = HEs to tick bites) in Comarca Lagunera, north of Mexico.

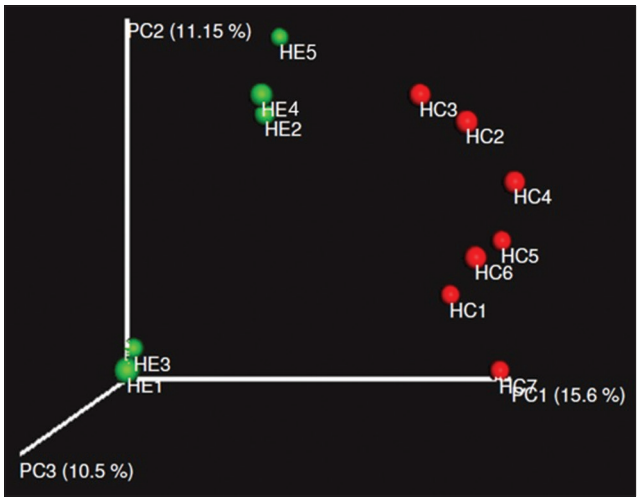


**Figure-2:** Principal coordinate analysis plot based on Bray-Curtis index representing blood samples of volunteers not exposed (HCs, red spheres) and exposed (HEs, green spheres) to tick bites in Comarca Lagunera, north of Mexico.

Staphylococcaceae ($\bar{x}$ = 3.41) (Figure-4). Finally, at the genus level, the relative abundance analysis indicated that *Anaerobacillus* ($\bar{x}$ = 10.04), a key phylum, PAC000748_g ($\bar{x}$ = 9.42), *Bacillus* ($\bar{x}$ = 8.96), and *Caldalkalibacillus* ($\bar{x}$ = 7.90) were predominant in the HCs, whereas in the HE group, *Kocuria* ($\bar{x}$ = 14.59), *Anaerococcus* ($\bar{x}$ = 3.51), *Staphylococcus* ($\bar{x}$ = 3.05), and *Treponema* ($\bar{x}$ = 2.93) were predominant (Figure-4). The complete list of bacteria found in this research at the phylum-to-species level is available in Table-S1.

The average global dissimilarity according to the SIMPER analysis at the phylum level was 45.15, the contribution of dissimilarity between populations for the taxon Firmicutes was 32.96%, and the difference was also significant (p = 0.014), as was that for Actinobacteria (p = 0.014) and Cyanobacteria (0.046). The average global dissimilarity at the family level was 79.07. The taxa that contributed the most to the dissimilarity were Bacillaceae (14.75%; p = 0.005), Halomonadaceae (2.30%; p = 0.034), and Spirochaetaceae, which exhibited a percent dissimilarity of 1.88% (p = 0.046). An average global dissimilarity of 85.97 at the genus level was recorded; the genera that contributed the most to the difference were *Kocuria* (8.42%; p = 0.005), *Anaerobacillus* (5.03%; p = 0.005), *Caldalkalibacillus* (3.17%; p = 0.009), *Treponema* (1.73%; p = 0.040), and *Clostridium* (1.19%; p = 0.009), among others (Table-2).

The biomarkers that differentiated the groups the most and showed a significant difference in the LEfSe

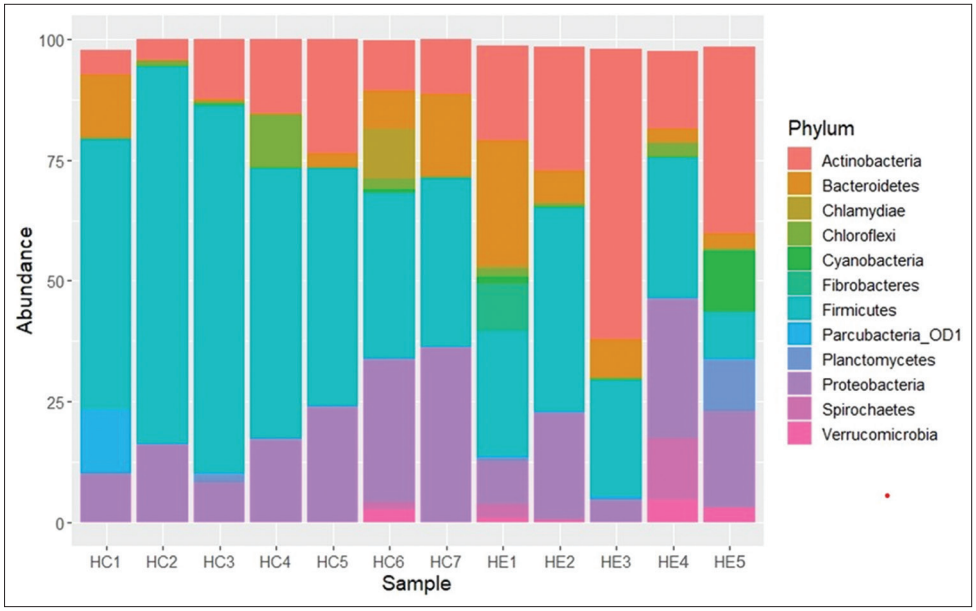


**Figure-3:** Relative abundance (%) at phylum level of the bacterial microbiota of human blood at Comarca Lagunera, north of Mexico (HCs = not exposed, HEs = exposed to tick bites).

LDA score in the HCs were the Firmicutes phylum, Bacillaceae family, Bacillales order, and Bacilli class, among others. In the HEs, these biomarkers were Actinobacteria phylum, Actinobacteria class, *Kocuria* genus, Spirochaetes phylum, Spirochaetia class, Cyanobacteria phylum, Spirochaetales order, and *Treponema* genus, among others (Figure-5).

## Discussion

The blood microbiome has been increasingly studied in an attempt to determine its meaning and role in humans. Although a core microbiome has not been found in human blood, dysbiosis related to certain pathologies has been highlighted in this environment. New molecular technologies such as NGS have facilitated the identification of bacteria in the blood of apparently healthy people [23]. In this study, we compared the blood of apparently healthy individuals who were exposed (HE) to a tick bite in the previous year to that of individuals who were never exposed to a tick bite (HC). Several studies have demonstrated the presence of a healthy blood microbiome or healthy HBM; therefore, it is also possible to evaluate abnormal changes or modifications (i.e., dysbiosis) in individuals who present with some type of pathology, such as cardiovascular diseases, inflammatory bowel disease, diabetes, asthma, or cirrhosis [7].

At present, there is still controversy, but it is difficult to clarify whether dysbiosis is a consequence of certain diseases or the cause of disease. However, there are no comparisons of individuals with apparently healthy HBMs with groups of people exposed to tick bites. Therefore, our working hypothesis focused on comparing and analyzing the differences between the bacterial profiles of the blood of humans exposed (HE) and those of humans not exposed (HC) to tick bites. Our research identified substantial differences in composition and demonstrated that the HE group had a greater abundance of sequences obtained in the analysis, as $\bar{x}$ = 57,253 sequences were assembled from a total of $\bar{x}$ = 148,394 sequences in the HC group, whereas in the HE group, $\bar{x}$ = 110,009 sequences were assembled from the obtained $\bar{x}$ = 214,263 sequences (Table-1). More than double the number of sequences was obtained in the HEs, even though the HEs had fewer analyzed individuals. In addition, the number of OTUs obtained in both populations also differed: 2774.4 in the HE population and 1892 in the HC population (Table-1).

No differences in alpha diversity were detected between the groups; however, a considerably high level of diversity was detected in both cases according to the Chao1, Shannon, and Simpson indices (Figure-1). In addition, a difference in beta diversity was detected using the Bray–Curtis index (Figure-2). This result indicates that the HE composition and the composition of healthy HBM microbiomes are different in terms of their configurations. Finally, according to the PCoA analysis, specific segregation occurred between the two groups (Figure-2). Although diversity was observed between the groups, the reason why one bacterial taxon was found in one group or another remains elusive. Some studies suggest that the presence of bacteria in the blood is due to translocations of other parts of the body, such as the intestinal tract, skin, and mucous membranes; moreover, some bacteria are vertically inherited by the mother [7, 24].

The most abundant phyla in the HC group were Firmicutes ($\bar{x}$ = 55.54%), Proteobacteria ($\bar{x}$ = 19.92%), Actinobacteria ($\bar{}$ = 11.69%), and Bacteroidetes ($\bar{x}$ = 5.95%) (Figure-3). The most common phyla reported in other studies on healthy HBM included the most dominant phyla, Proteobacteria, followed by Actinobacteria, Firmicutes, and

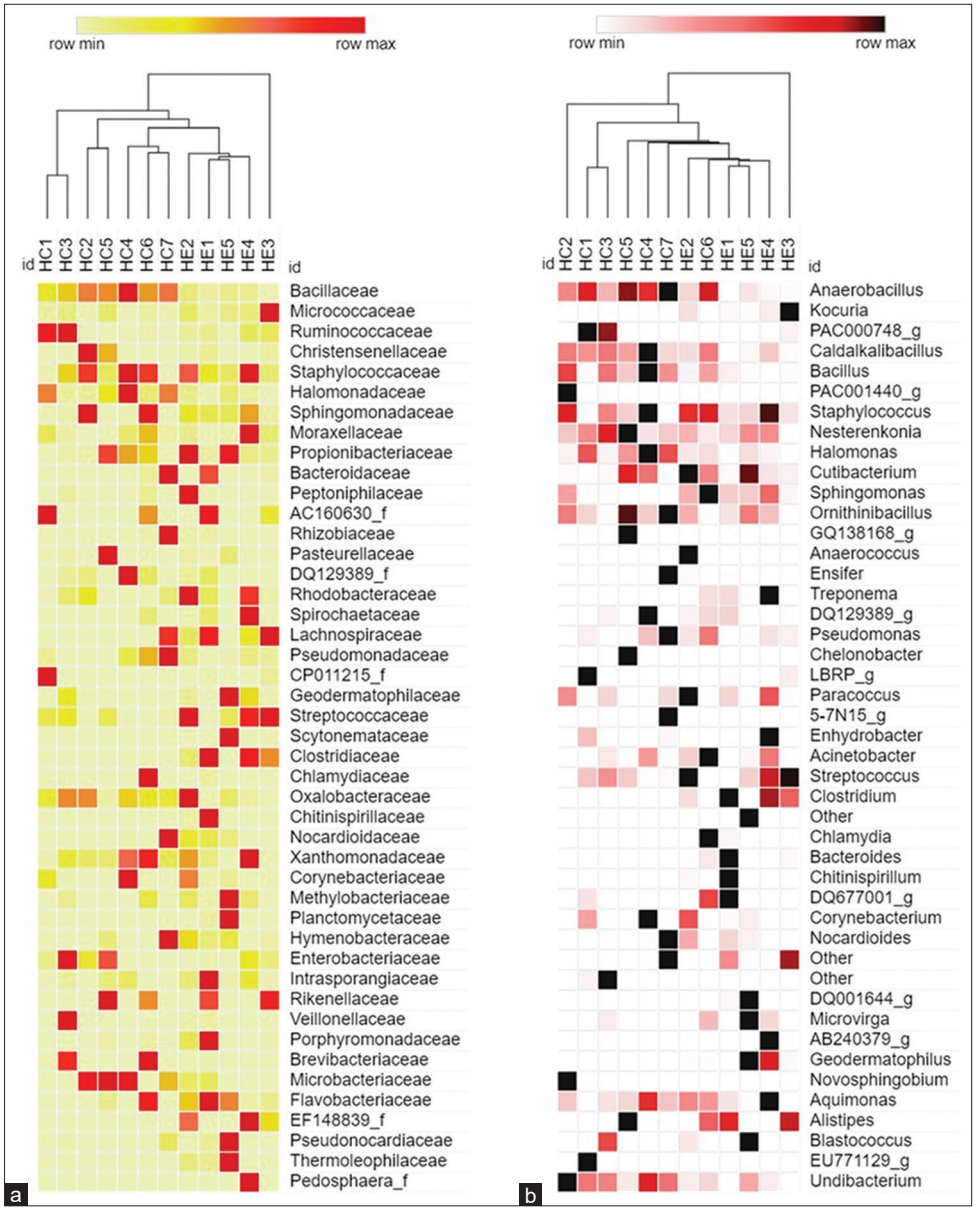


**Figure-4:** Heatmaps for family (a) and genera (b) levels from the bacterial microbiota of human blood at Comarca Lagunera, north of Mexico (HCs = not exposed, HEs = exposed to tick bite).

Bacteroidetes [24–26]. Panaiotov *et al*. [27] identified Proteobacteria as the most abundant phylum in blood samples from healthy individuals, comprising 93% in non-cultured samples and 46% in cultured samples. Similarly, Actinobacteria and Firmicutes, comprising 2%, were identified among the noncultivated samples. Among the cultured samples, 25% were Firmicutes, 14% were Actinobacteria, 6% were Bacteroidetes, 3% were Fusobacteria, and 2% were Cyanobacteria [27]. Previously, an overrepresentation of the Proteobacteria phylum was reported in healthy humans (i.e., 73.4%) or in individuals with some type of disorder, such as bipolar disorder, schizophrenia, amyotrophic lateral sclerosis, or a control group [28]. These findings coincide with the microbiome of other parts of the human body, mainly the oral cavity and intestine; therefore, these bacteria are likely to have translocated from these areas [28, 29]. In 2016, Païssé *et al*. [24] analyzed different fractions of human blood and found that Proteobacteria and Firmicutes were the most abundant phyla in whole blood.

The genus *Bacillus* has been detected in both plasma and erythrocytes in the blood of donors [30]. In our study, the *Bacillus* genus was also one of the most common genera in the HC group and was highly significant concerning the observed dissimilarity between the two groups (Table-2) as well as the biomarkers (Figure-5). However, other phyla were more abundant in the bacterial profiles of healthy individuals. In 2008, Moriyama *et al*. [31] identified a set of bacterial taxa comprising *Bacillus*, *Flavobacteria*, *Stenotrophomonas*, and *Serratia* in the blood of two healthy individuals.

The genus *Ehrlichia* within the Anaplasmataceae family was found in only 20% of the HE group. This genus is reported in the blood of people with suspected TBD [32], and it was the only genus classified as TBD

**Table-2:** Percentage similarity analysis (SIMPER) considering the AVD of the bacteria in the blood at phyla, family, and genera levels from two groups of volunteers not exposed (HCs) and exposed (HEs) to tick bites in Comarca Lagunera, north of Mexico.

| Taxon | AVD | Contribution % | Cumulative % | Mean HCs | Mean HEs | U | p |
|---|---|---|---|---|---|---|---|
| Phylum | | | | | | | |
| Firmicutes | 14.88 | 32.96 | 32.96 | 0.552 | 0.263 | 2.0 | 0.014* |
| Actinobacteria | 10.47 | 23.20 | 56.15 | 0.119 | 0.322 | 2.0 | 0.014* |
| Proteobacteria | 5.46 | 12.11 | 68.26 | 0.201 | 0.169 | 14.0 | 0.626 |
| Bacteroidetes | 4.26 | 9.44 | 77.71 | 0.059 | 0.092 | 13.0 | 0.515 |
| Spirochaetes | 1.60 | 3.55 | 81.27 | 0.002 | 0.031 | 14.0 | 0.623 |
| Cyanobacteria | 1.39 | 3.08 | 84.35 | 0.001 | 0.027 | 6.0 | 0.046* |
| Family | | | | | | | |
| Bacillaceae | 11.66 | 14.75 | 14.75 | 0.294 | 0.060 | 0.0 | 0.005* |
| Micrococcaceae | 7.00 | 8.85 | 23.61 | 0.042 | 0.174 | 7.0 | 0.104 |
| Ruminococcaceae | 6.20 | 7.84 | 31.45 | 0.109 | 0.038 | 13.5 | 0.562 |
| Christensenellaceae | 4.67 | 5.91 | 37.36 | 0.088 | 0.011 | 17.0 | 1.000 |
| Peptoniphilaceae | 2.09 | 2.64 | 40.00 | 0.003 | 0.039 | 13.0 | 0.468 |
| Halomonadaceae | 1.82 | 2.30 | 42.31 | 0.041 | 0.006 | 4.0 | 0.034* |
| Sphingomonadaceae | 1.79 | 2.26 | 44.57 | 0.026 | 0.023 | 10.0 | 0.252 |
| Moraxellaceae | 1.67 | 2.12 | 46.69 | 0.015 | 0.031 | 16.5 | 0.935 |
| Bacteroidaceae | 1.65 | 2.08 | 48.78 | 0.019 | 0.019 | 16.5 | 0.923 |
| Staphylococcaceae | 1.52 | 1.92 | 50.71 | 0.034 | 0.034 | 17.0 | 1.000 |
| Spirochaetaceae | 1.49 | 1.88 | 52.59 | 0.002 | 0.029 | 6.0 | 0.046* |
| Propionibacteriaceae | 1.36 | 1.72 | 54.32 | 0.015 | 0.027 | 9.0 | 0.193 |
| Rhodobacteraceae | 1.25 | 1.59 | 55.91 | 0.005 | 0.026 | 10.0 | 0.252 |
| Rhizobiaceae | 1.24 | 1.57 | 57.48 | 0.023 | 0.002 | 17.0 | 1.000 |
| Pasteurellaceae | 1.22 | 1.54 | 59.03 | 0.021 | 0.004 | 10.5 | 0.259 |
| †Anaplasmataceae | 0.01 | 0.01 | 99.69 | 0.000 | 0.000 | 14.0 | 0.310 |
| Genus | | | | | | | |
| *Kocuria* | 7.24 | 8.42 | 8.42 | 0.000 | 0.146 | 0.0 | 0.005* |
| PAC000748_g | 4.78 | 5.56 | 13.99 | 0.094 | 0.003 | 15.0 | 0.669 |
| *Anaerobacillus* | 4.32 | 5.03 | 19.03 | 0.099 | 0.013 | 0.0 | 0.005* |
| *Bacillus* | 4.18 | 4.86 | 23.90 | 0.089 | 0.007 | 2.0 | 0.014* |
| PAC001440_g | 3.01 | 3.50 | 27.40 | 0.060 | 0.000 | 14.0 | 0.562 |
| *Caldalkalibacillus* | 2.73 | 3.17 | 30.58 | 0.078 | 0.024 | 1.0 | 0.009* |
| *Halomonas* | 1.82 | 2.11 | 32.70 | 0.041 | 0.006 | 4.0 | 0.034 |
| *Anaerococcus* | 1.73 | 2.01 | 34.71 | 0.000 | 0.034 | 10.5 | 0.104 |
| *Staphylococcus* | 1.57 | 1.82 | 36.54 | 0.034 | 0.030 | 16.0 | 0.870 |
| *Treponema* | 1.49 | 1.73 | 38.27 | 0.002 | 0.029 | 6.0 | 0.040* |
| GQ138168_g | 1.37 | 1.60 | 39.88 | 0.027 | 0.000 | 15.0 | 0.498 |
| *Nesterenkonia* | 1.36 | 1.59 | 41.47 | 0.038 | 0.023 | 14.0 | 0.626 |
| *Cutibacterium* | 1.34 | 1.56 | 43.04 | 0.015 | 0.026 | 9.5 | 0.222 |
| *Sphingomonas* | 1.33 | 1.55 | 44.59 | 0.017 | 0.021 | 9.0 | 0.185 |
| *Ensifer* | 1.19 | 1.38 | 45.98 | 0.023 | 0.001 | 15.5 | 0.771 |
| *Enhydrobacter* | 1.12 | 1.30 | 47.28 | 0.003 | 0.020 | 15.0 | 0.728 |
| *Clostridium* | 1.11 | 1.29 | 48.58 | 0.000 | 0.022 | 3.5 | 0.009* |
| Other | 1.11 | 1.29 | 49.87 | 0.000 | 0.022 | 14.0 | 0.310 |
| *Chelonobacter* | 1.04 | 1.21 | 51.08 | 0.020 | 0.000 | 15.0 | 0.498 |
| *Chitinispirillum* | 1.02 | 1.19 | 52.27 | 0.000 | 0.020 | 7.0 | 0.033* |
| DQ129389_g | 1.01 | 1.18 | 53.46 | 0.018 | 0.005 | 17.0 | 0.862 |
| LBRP_g | 1.01 | 1.17 | 54.63 | 0.018 | 0.002 | 14.0 | 0.522 |
| *Bacteroides* | 0.99 | 1.15 | 55.79 | 0.001 | 0.019 | 13.0 | 0.393 |
| *Ornithinibacillus* | 0.94 | 1.09 | 56.89 | 0.022 | 0.012 | 14.0 | 0.626 |
| †*Ehrlichia* | 0.01 | 0.01 | 98.82 | 0.000 | 0.000 | 14.0 | 0.310 |

*Indicates significant diff, *Difference in dissimilarity between groups, †Indicates the taxa considered as TBP (Tick-borne pathogens), AVD: Average of dissimilarity, Contrib. %: Contribution of dissimilarity in percentage, Cum. %: Cumulative in percentage, U: U Man–Whitney test, p: p value

found in the present study. In addition, several potentially pathogenic bacterial phylotypes were found in both experimental groups, including *Pseudomonas*, *Staphylococcus*, *Kocuria*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Staphylococcus*, *Klebsiella*, *Treponema*, *Streptococcus*, *Escherichia*, *Salmonella*, *Fusobacterium*, *Moraxella*, and *Mycoplasma*. The genus *Kocuria* was the most abundant in the HEs among the HCs. *Kocuria* was also the genus that most differentiated the two populations according to the SIMPER analysis (8.42%), and the dissimilarity was highly significant (p = 0.005) (Table-2). These results are consistent with an investigation in the same region of the study area (i.e., Comarca Lagunera) and with the same methodology used in the present work, where the genus *Kocuria* was found to be the most abundant in the blood of dogs (*Canis lupus familiaris*) infested with ticks ($\bar{x}$ = 6%) at relative abundance (Mejia-García

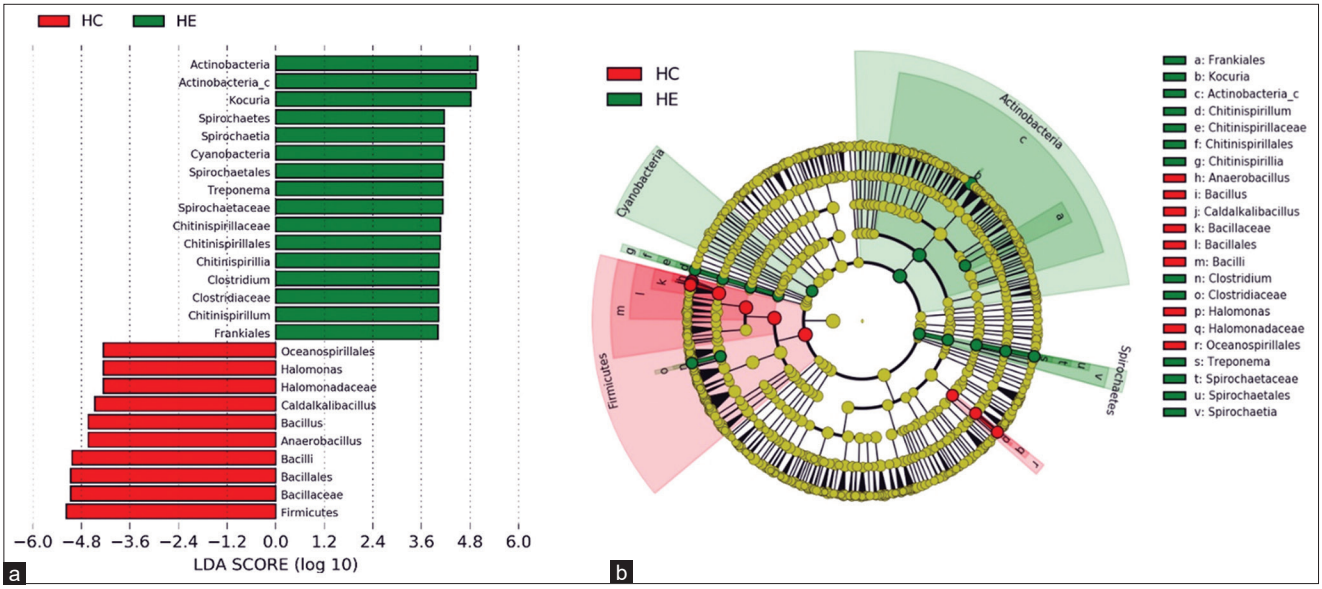


**Figure-5:** LEfSe analysis from the bacterial microbiota of human blood (volunteers not exposed [HC] and exposed [HE] to tick bites) in Comarca Lagunera, north of Mexico. (a) Bar graph shows LDA scores which indicates the taxonomic key for differentiation in both groups. (b) The cladogram generated by LEfSe indicates the main biomarkers between groups. Each successive circle represents taxonomic levels. Red-colored regions indicate taxa enriched in the HCs, while green-colored regions indicate taxa enriched in the HEs. LEfSe=Linear discriminant analysis effect size, LDA=Linear discriminant analysis.

*et al*. 2022., unpublished data); it was also a biomarker that differentiated both groups according to the LEfSe analysis (Figure-5). *Kocuria* is related to clinical cases of different indoles and to enzymatic processes because of the production of proteases and catalases as well as volatile compounds in food. In addition, the production of biofilms and their resistance to antibiotics makes members of the *Kocuria* genus potentially undesirable [33]. Furthermore, several species of the genus *Kocuria* have been shown to cause various diseases, including sepsis, infective endocarditis, meningitis, cholecystitis, urinary tract infections, catheter-linked bacteremia, peritonitis, and abscesses [34–36].

The diagnosis of *Kocuria* has been undervalued mainly because of misidentifications by *staphylococci* and limited biochemical tests and automatic identification systems. Although the virulence of the genus is not well established, it is considered an important pathogen in human medicine and a recurrent bacteremia genus [36]. Moreover, different studies have shown *Kocuria* within the internal microbiome of ticks [37–39]. Li *et al*. [39] identified *Kocuria* as the most isolated genus of samples from the midgut of ticks by sequencing the 16S DNA gene. The intestines are the structures that have the first contact with pathogens within the vector. The pathogens then migrate to other parts of the body of the tick and play a key role in the transmission and prevalence of infectious tick diseases. Certain factors must be considered with regard to the possibility of the genus *Kocuria* as an opportunistic organism that can trigger a clinical response in immunosuppressed people [39].

While *Treponema* was found in 80% of the HEs and was observed in only 20% of the HCs, it was one of the most abundant genera in our study. *Treponema* ($\bar{x}$ = 2.93), represented graphically on the heatmap relative abundance and SIMPER analysis, had the greatest contribution to the dissimilarity between groups(Table-2). This genus has been identified as a bacterium transmitted by transfusions of people exposed to ticks [40]. In addition, *Treponema* has been identified in the analysis of bacterial profiles in ticks [41] and is related to individuals that are seropositive for Lyme disease caused by *Borrelia burgdorferi* [42]. There are very marked similarities in tick-borne spirochetes, both in terms of the morphological and protein nature of the diseases they cause [43]. Despite these similarities, the most common disease caused by these spirochetes is attributed to the etiological agent *Treponema pallidum*, which causes venereal syphilis. Although we did not find this species, we observed EU463520_s and the *Treponema* species *Treponema brennaborense*; the latter could be a new species described in the blood of humans that has previously been associated by Buyuktimkin *et al*. [44] with bovine digital dermatitis, periodontal diseases, and skin diseases in humans and animals [44].

Kingry *et al*. [32] found Spirochaetales and Rickettsiales to be the most representative in the blood of donors suspected of having some TBDs, and in the present study, the LEfSe analysis indicated the same relationship (Figure-5). Importantly, the presence of spirochetes in the blood is not indicative of disease unless the patient has clinical signs [45]. Nonetheless, despite non-apparent clinical signs at the time of sampling, all the people in the study were exposed to tick bites and had symptoms at the time of exposure; symptoms such as headache, fever, and myalgias were also described by the people under analysis.

Methods for detecting pathogens by sequencing have several limitations, especially with regard to samples with very low biomass, such as blood, where some environmental or laboratory contaminants are certainly common, especially nonpathogenic taxa, such as *Bradyrhizobium* [46], which was detected in only a single sample in the present study. However, the superposition of contaminating taxa with pathogens or possible pathogens does not occur in bacterial analyses derived from vectors. Moreover, directed metagenomics has been proposed as a recommended analysis method for tick-borne pathogens rather than other bacterial infections [47], especially because it is possible to identify potentially pathogenic bacteria that have not yet been reported [32].

The complexity of obtaining an accurate diagnosis is a disturbing concern regarding TBD, mainly because these diseases have nonspecific clinical signs that are very similar. Certainly, in most cases, these diseases are self-limiting. However, in the case of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *and R. rickettsii*, infections can potentially be fatal, particularly in elderly immunocompromised children or people with comorbidities. Children under 10 years of age are the most affected [48]. RMSF is the most common rickettsial disease in Mexico, with lethal outbreaks occurring in several northern states (i.e., Coahuila, Durango, and Sonora) [9]. In our study, unlike other scientific and press publications where a high incidence and mortality of RMSF due to *R. rickettsii* have been reported, RMSF was not detected in people exposed to tick bites. RMSF is endemic to Comarca Lagunera, and RMSF has been detected both in studies involving people and in studies involving dogs and their main ectoparasites (*R. sanguineus*) using different methods both in Mexico and in the region under study [8, 9, 49, 50]. The mortality rate of RMSF in the state of Coahuila was 14.5% in 2012, 50% in 2017, 50% in 2020, 60% in 2021, and 69% in 2022, the highest in the past 10 years. The Secretary of Health reported 231 cases, 29 of which were confirmed, and 20 of these patients died, representing 69% of the fatalities. The RMSF problem is worsened because 60%–75% of cases are misdiagnosed, and appropriate care is not provided in a timely manner [10, 51]. However, the data provided do not rule out the possibility that these individuals faced bacterial coinfections because of tick bites and not only because of the genus *Rickettsia*. Therefore, more robust studies of bacterial detection by molecular techniques are required to provide additional epidemiological information on TBD in the region because simple PCR, as a diagnostic technique, is aimed at a particular pathogenic organism and can lead to misdiagnosis.

## Conclusion

This is the first study to compare bacteria in the blood of healthy people exposed to a tick bite versus unexposed people using next-generation massive sequencing. Our study confirmed that the blood of healthy individuals is colonized by a considerable number of bacteria. In addition, our study detected significant differences in diversity and microbiological composition in both experimental groups, while *Ehrlichia* was identified as the only TBD in the HE groups.

Different pathogens, such as *Chlamydia*, *Clostridium*, *Kocuria*, *Staphylococcus*, and *Treponema*, were also observed in the HE group rather than in the HC group. Undoubtedly, we still have fragmentary knowledge about the main microbiome interactions between humans and the main ectoparasites of neglected dogs; future studies must be developed to better understand the possible association between blood dysbiosis and people bitten by ticks.

## Supplementary Materials

Table-S1: Relative abundance of bacterial taxa in 12 blood samples from people, five people exposed to tick bites and seven people not exposed to tick bites in Comarca Lagunera. The sequences used in this study are deposited in the NCBI web accession number (BioProject PRJNA908861).

## Authors' Contributions

SIBG: Designed and conceptualized the study, Sample collection, analyzed the data and wrote the manuscript; CGDP and CAMH: Conceptualized, reviewed and edited the manuscript; QKSR and LMVN: Sample preparation and DNA extraction. FVP, CDV, and ADCM: Acquisition of resources and data collection. All authors have read, reviewed, and approved the final manuscript.

## Acknowledgments

We thank the people who kindly provided their blood sample for the present study. We appreciate the funding provided by Department of Conservation Medicine, Faculty of Biological Sciences, Juarez University of the State of Durango (Grant 2019-LMC2) and National Health Laboratory: Molecular Diagnosis and Environmental Effect on Chronic-Degenerative Diseases, Faculty of Higher Studies Iztacala, National Autonomous University of Mexico. Special support provided by the Chapingo Autonomous University, Regional University Unit of Arid Zones.

## Competing Interests

The authors declare that they have no competing interests.

## Publisher's Note

Veterinary World (Publisher of International Journal of One Health) remains neutral with regard to jurisdictional claims in published institutional affiliation.

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