Portraying the gut bacterial communities and blood feeding sources of triatomine bugs (Hemiptera: Reduviidae), vectors of Chagas disease

Investigadora principal
Luisa María Arias Giraldo

Investigadores asociados
Claudia Marina Muñoz
Diana Carolina Hernández Castro
Giovanny Andrés Herrera Ossa
Valentina Caicedo Garzón
Natalia Velásquez Ortiz
Omar Cantillo
Plutarco Urbano

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Estudiante:
Luisa María Arias Giraldo

Director de tesis:
Juan David Ramírez González

Facultad de Ciencias Naturales y Matemáticas
Programa de Biología
Universidad del Rosario

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TESIS

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Nombre de la investigadora: Luisa María Arias Giraldo, estudiante del programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, luisam.arias@urosario.edu.co

Nombre del director del proyecto: Juan David Ramírez González, microbiólogo, Profesor Asociado de la Universidad del Rosario. Ph.D. en Ciencias Biológicas y M.Sc. en Ciencias Biológicas, Universidad de los Andes. Pregrado en Microbiología, Universidad de los Andes – juand.ramirez@urosario.edu.co

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Luisa M. Arias-Giraldo[1], Marina Muñoz[1], Carolina Hernández[1], Giovanny Herrera[1], Valentina Caicedo-Garzon[1], Natalia Velásquez Ortiz[1], Omar Cantillo[2], Plutarco Urbano[3], Juan David Ramírez[1*]

[1] Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia.

[2] Grupo de Biología Molecular de Enfermedades Infecciosas, Universidad de Antioquia, Medellín, Colombia.

[3] Fundación Universidad del Trópico Americano (Unitropico), Yopal, Colombia.

Corresponding author: Juan David Ramírez

Address: Carrera 24 # 63C - 69, Bogotá, Colombia

Telephone number: +57-1-297 0200 Ext: 4033

e-mail: juand.ramirez@urosario.edu.co

Abstract

BACKGROUND: Triatomine bugs (Hemiptera: Reduviidae) are insects that play an important role as vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease. This role can be affected by several aspects, like the gut bacteriome and feeding sources of the insect. Little is known about this bacteriome and the changes it can undergo when *Trypanosoma cruzi* is present or the feeding source of the insect changes. Here, we conducted

a first characterization of the bacteriome of the main triatomine genera in Colombia (*Panstrongylus, Rhodnius* and *Triatoma*) and included *Psammolestes* due to its recent evidence of *T. cruzi* infection.

METHODS: We collected 55 triatomine bugs corresponding to 4 genera (*Panstrongylus, Psammolestes, Rhodnius* and *Triatoma*) and 6 species (*P. geniculatus, Ps. arthuri, R. pallescens, R. prolixus, T. maculata* and *T. venosa*) in eight departments of Colombia. DNA from gut was extracted and *T. cruzi* and DTUs were detected using qPCR and conventional PCR, respectively. PCR products for vertebrate 12S rRNA gene and bacterial 16S gene were pooled, sequenced on an Illumina Miseq flow-cell and processed using QIIME2 software. Complementary sequences obtained through Sanger sequencing were submitted to BLASTn as well for feeding sources identification. Inverse Simpson index was calculated, and we made a PCA and visual representations of the data using CIRCOS tool.

RESULTS: *T. cruzi* was successfully detected in 76.4% (*n*=42) of the samples. Twenty-seven bacterial phyla were identified and the most abundant of these were *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*, which accounted for over 90% of the bacteriome and were significantly different between triatomine species. Twenty-one vertebrate feeding sources were identified and the most abundant were *Homo sapiens* (53.86%), *Equus caballus* (21.71%) and *Coendou melanurus* (10.34%). The feeding behaviour was significantly different between triatomine species and, despite not statistically significant, there were also differences between *T. cruzi* negative and positive samples.

CONCLUSIONS: Understanding the microbiota present in vectors of human-borne diseases is pivotal for the comprehension of the relationships with the parasites they transmit, and the other animals involved in the life cycle of the parasite. The methodology used in this study yielded a higher diversity than reported before, in both bacterial taxa and feeding sources.

Microbial composition of triatomine gut seems to depend primarily on the triatomine species and the ecotope even though some differences can be observed when *T. cruzi* is present and the feeding source changes.

Keywords: Next-generation sequencing, bacteriome, Triatominae, *Trypanosoma cruzi*, feeding source.

Introduction

Triatomines (Hemiptera: Reduviidae) are insects that play an important role as vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease [1]. This parasite presents tremendous genetic diversity and has been divided into six Discrete Typing Units (DTUs) from TcI to TcVI [2]. Upon their entrance to the insect, these parasites settle in the gut where they replicate and later differentiate (metacyclogenesis) and proliferate [3]. Almost all the life cycle of *T. cruzi* occurs in the gut of the triatomine, given that transformation of trypomastigotes in epimastigotes and division of these epimastigotes take place there [4]. Another trypanosomatid named *T. rangeli* can also infect triatomines [5]. *T. rangeli*, apart from multiplying in the gut of the insect vector, also invades the hemolymph to complete its development in the salivary glands of the triatomine [4]. Consequently, both parasites colonize the triatomine gut at some point of their development. Therefore, the bacteriome present in the triatomine gut might determine its ability to get, maintain, replicate, and/or transmit parasites such as *T. cruzi* and *T. rangeli* [6, 7].

Also, it is known that gut microbiota affects the development of *T. cruzi* inside the vector[4, 8–11]. This modulation of the parasite and its pivotal significance in the life cycle of the parasite [12] makes triatomines important in the control of Chagas disease. Triatomines belong to the subfamily Triatominae (Hemiptera: Reduviidae), composed by approximately

152 extant species present in the Americas [13]. The main triatomine vectors in the Americas belong to the genera *Rhodnius*, *Triatoma* and *Panstrongylus* [1, 14]. In Colombia, 15 of 24 reported species are naturally infected by *T. cruzi*, and the main vector species are *R. prolixus*, *T. dimidiata* and *P. geniculatus*, which can be found in nearly all the country [15]. *Psammolestes*, on the other hand, despite being considered as a genus associated with bird nests, has been recently found naturally infected with *T. cruzi*, therefore more studies about this genus are needed [16, 17]. Nevertheless, the bacteriome of the gut of these insects has not been profoundly studied, therefore multiple aspects remain to be explored.

Da Mota et *al.* (2012) found that the gut bacteriome of *Rhodnius*, *Triatoma* and *Panstrongylus* has low diversity with only one predominant phylum (*Actinobacteria*) [18], which was later confirmed by other studies [19–21]. Díaz et *al.* (2016) showed that the diversity of the gut microbiota increases when *T. cruzi* is present [20], which was also found in posterior studies [21, 22]. Additionally, in these studies a trend in which the bacteriome changes depending on the triatomine species has been observed. Dumonteil et *al.* (2018) found that the 23 bacterial orders present in *T. dimidiata* change when the feeding source is distinct and that a difference between sexes exists. Of these bacterial orders, the ones that showed the highest abundance were *Bacillales*, *Actinomycetales*, *Enterobacteriales* and *Burkholderiales*. More importantly, in this study it was found that one triatomine can feed from multiple hosts, which suggests frequent host changes that can affect the transmission dynamics of *T. cruzi* and bacterial communities in triatomine´s gut [23].

Despite these studies, the complete knowledge of this topic is scarcely unknown given the limitations in terms of the countries where this has been evaluated and the few triatomine species that have been used. Moreover, to date, only a small part of these characterizations has been done in Colombia, one of the countries with the highest incidence

of Chagas disease [24]. Consequently, in this country, it is still unknown how the infection rate of *T. cruzi* affects the bacteriome of the insect. Additionally, even though the range of triatomine feeding sources is wide [23, 25–28] and it is known to affect the microbiota [20, 23, 29]. This aspect has not been vastly evaluated to completely understand the dynamics involved. Also, only a few studies have been made using next generation sequencing (NGS) techniques [22, 23, 30] despite its capacity for revealing multiple consumed species simultaneously and characterizing many more samples than traditional techniques [31].

Therefore, herein we conducted a robust and first characterization of the gut bacteriome of the main triatomine genera in Colombia (*Panstrongylus, Rhodnius* and *Triatoma*) and included *Psammolestes* due to its recent evidence of *T. cruzi* infection. We compared the gut bacteriome between insects (i) naturally infected and not infected with *Trypanosoma cruzi*, and (ii) with different feeding habits.

Methods

Insect sampling and dissection

We collected 55 triatomine bugs (Table S1) corresponding to 4 genera (12 *Panstrongylus geniculatus, 7 Psammolestes arthuri,* 8 *Rhodnius pallescens* and 22 *R. prolixus,* 3 *Triatoma maculata* and 3 *T. venosa*) from 2012 to 2018 in different departments of Colombia (Arauca, Bolívar, Boyacá, Casanare, La Guajira, Magdalena, Meta and Santander) (Fig. 1). The insects were collected using different entomological surveillance techniques for each ecotope (domestic, peridomestic and sylvatic), which have been described elsewhere [32]. Bugs were kept in 100% ethanol in Eppendorf tubes and upon arrival they were stored at -20 °C until the moment of dissection. The dissections performed

resulted in two tubes per insect: (i) one with head, legs and sniper, and (ii) one with the abdominal region of the insect, being these latter tubes, the ones used in this study after being washed three times with PCR ultra-pure water.

Detection and genotyping of T. cruzi/T. rangeli

DNA from the gut was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germany) with minor modifications of the manufacturer's protocol. DNA concentrations were determined on a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

The presence of *T. cruzi* parasites in bugs was detected by qPCR with primers Cruzi1 (ASTCGGCTGATCGTTTTCGA) and Cruzi2 (AATTCCTCCAAGCAGCGGATA) and probe Cruzi3 (CACACACTGGACACCAA) as described elsewhere [27, 33]. The results were considered positive when the amplification exceeded the threshold of fluorescence 0.01. For the insects with positive results by qPCR, it was necessary to identify if the presence detected was due to *T. cruzi* or *T. rangeli* parasites, therefore a kinetoplast fragment DNA amplification was performed using primers 121 (AAATAATGTACGGGKGAGATGCATGA) and 122 (GGTTCGATTGGGGTTGGTGTAATATA) as described elsewhere [34]. For the insects identified as positive for *T. cruzi*, the DTU of the parasite was identified using the algorithm implemented by Hernández et *al.*, 2016 [27].

Feeding sources and bacterial communities identification

Amplicon sequencing was performed by Novogene: Genome Sequencing Company (Beijing, China). PCR products for vertebrate 12S rRNA gene and bacterial 16S gene were

pooled and 2 x 300 paired-end sequenced on an Illumina Miseq flow-cell (Illumina, San Diego, USA). For the amplification of the V4 region of bacterial rRNA 16S, the universal primers used were 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), which amplify a sequence fragment of 390 bp [35]. The reads used in the posterior analyses were obtained from each insect after denoise and clustering processes using QIIME 2 [36], which assures the reduction of sequence errors. The PCR products used for vertebrate 12S rRNA were obtained through the amplification of a 215 bp fragment of the 12S rRNA gene using primers L1085 (5′-CCCAAACTGGGATTAGATACCC-3′) and H1259 (5′-GTTTGCTGAAGATGGCGGTA-3′) [23]. For the identification of blood feeding sources in 10 samples that were unfit for NGS (the requirements of DNA volume and concentration were not reached), Sanger sequencing method was used for PCR amplification of the 215 bp fragment of the 12S rRNA mentioned earlier, using the algorithm described elsewhere [37, 38]. The resulting sequences were later submitted to BLASTn with a threshold of 90% sequence identity.

Bioinformatic analysis – bacteriome data

Sequences of 16S gene were used for identification of bacteria. We made sure the sequences produced by Ilumina Miseq were cleaned by extracting the barcodes attached, demultiplexing the files and removing the sequencing noise. With these clean sequences, we made a taxonomic identification of bacteria. Operational Taxonomic Units (OTUs) were defined at the level of 97% similarity against the Ribosomal Database Project (RDP) Classifier reference sequences [39], obtaining one representative sequence for each OTU. These representative sequences were aligned using PyNAST [40] and the taxonomy assignment was made using the uclust consensus taxonomy assigner [41]. The taxonomical

classification was performed down to species level, nonetheless, the two levels considered in this study were phylum and genus. However, given the amount of data, bacteria from those two levels that were detected with less than 3% of abundance in the bacteriome of an individual triatomine, were discarded in the analysis. The number of reads obtained for each taxon was used as a proxy of its abundance, as previously reported [23].

To assess sequence diversity, we measured alpha diversity calculating inverse Simpson diversity index, and beta diversity constructing a Principal Component Analysis (PCA) with R, using the package "factoextra". Additionally, we calculated Margalef richness index. We also constructed rarefaction curves to estimate species richness of our sampling. All the above, except the PCA, were performed using QIIME 2 software. Additionally, we used the online tool CIRCOS (http://circos.ca/) to graphically represent the relative abundance and distribution of bacteriome components in triatomines per species and per infection state for both cases [42].

Bioinformatic analysis – feeding sources data

12S gene sequences were used to describe the feeding sources preferences of triatomines. We made sure the sequences produced by Ilumina Miseq were cleaned by extracting the barcodes attached, demultiplexing the files and removing the sequencing noise. With these clean sequences, we made a taxonomic identification of feeding sources. In the case of this set of data, there was no reference database available in QIIME2 software, therefore we mapped the obtained sequences with Burrows-Wheeler Aligner method (BWA) against a reference dataset made by us of more than 250 vertebrate mitochondrial sequences, one per vertebrate. This dataset was made performing an intensive search in NCBI, which targeted the mitochondrial genome of each vertebrate species selected. Once found, we

looked for the primers (L1085 and H1259) sequences and extracted the resulting sequence that was positioned between these two primers sequences, adding them to a fasta file. Therefore, this dataset included a 215 bp fragment of 118 mitochondrial sequences of humans (n = 1, since it is only one species), reptiles (n = 8), birds (n = 59) and sylvatic and domestic mammals (n = 47) that are known as feeding sources for several triatomines and related (Table S2) [23, 27, 28, 43].

Additionally, we used the online tool CIRCOS (http://circos.ca/) to graphically represent the relative abundance and distribution of feeding sources in triatomines per species and per infection state for both cases [42]. The number of reads obtained for each species was used as a proxy of its abundance, as reported elsewhere [23].

Statistical analyses

Variables such as triatomine species, ecotope (Domestic, peridomestic, sylvatic), presence of *T. cruzi*, bacterial taxa composing the gut bacteriome of triatomines, and triatomine feeding sources were initially treated as categoric variables. The association between these variables was evaluated using Pearson Chi-Squared tests. Odds ratio (ORs), with its corresponding IC95%, were calculated to measure the strength of association between the analysed variables.

Results

Detection and genotyping of T. cruzi

The overall *T. cruzi* infection frequency was 76.4% (n = 42). The frequency of *T. cruzi* infection within each species was the following: *P. geniculatus* 83.33% (10/12), *Ps.*

arthuri 57.14% (4/7), *R. prolixus* 63.64% (14/22). In the cases of *R. pallescens*, *T. maculata* and *T. venosa* the frequency of infection was 100%. Additionally, the only insect found with a coinfection between *T. cruzi* and *T. rangeli* was *R. prolixus*.

TcI and TcII-TcVI were detected, being TcI the predominant DTU (66.7%, *n*= 28). Between the positive samples for this DTU we found six *P. geniculatus* (21.43%), three *Ps. arthuri* (10.71%), seven *R. pallescens* (25%), eight *R. prolixus* (28.57%), one *T. maculata* (3.57%) and three *T. venosa* (10.71%). The only case detected of TcII-TcVI (2.4%) belonged to a *T. maculata* insect. We also found six (14.3%) mixed infection cases (TcI and TcII-TcVI), which corresponded to four *P. geniculatus*, one *Ps. arthuri* and one *R. prolixus*. We found seven ND (Not detectable) cases (16.6%) which corresponded to one *R. pallescens*, five *R. prolixus* and one *T. maculata*. Within the cases detected as TcI, TcIDom and TcISylv were found. We detected nine (32.1%) TcIDom, of which two (22.22%) corresponded to *P. geniculatus*, one (11.11%) to *Ps. arthuri*, four (44.44%) to *R. pallescens*, one (11.11%) to *R. prolixus*, and one (11.11%) to *T. venosa*. Likewise, the four cases detected as TcISylv (14.3%), belonged to *R. prolixus* samples. We also detected six (21.4%) mixed infection cases, where TcIDom and TcISylv are found together in a single sample, and they were composed of three *P. geniculatus*, two *Ps. arthuri* and one *R. prolixus*. The ND cases detected were nine (32.1%) and corresponded to one *P. geniculatus*, three *R. pallescens*, two *R. prolixus*, one *T. maculata* and two *T. venosa*. ND results correspond to cases in which the DTU could not be determined because there was not DNA available for the PCR or, despite being a *T. cruzi* positive result, the parasitic load seemed to be too low to be typed. This applies for ecotope information as well.

Bacterial communities identification

Approximately 500,000 sequences were analysed and a total of 27 bacterial phyla were identified with a threshold of high confidence (97%) (Fig. 2). Rarefaction curves indicated that a great part of the richness was identified with our sampling (Fig. S1). The most abundant phyla were *Proteobacteria* (33.92%), *Actinobacteria* (32.41%), *Firmicutes* (19.59%) and *Bacteroidetes* (7.56%), which together accounted for over 90% of the gut bacteriome identified. Therefore, these four phyla were the main target for the identification of bacterial genera (Fig. 3), which lead to the following: 14 genera were identified for *Actinobacteria*, 18 for *Bacteroidetes*, 14 for *Firmicutes*, and 13 for *Proteobacteria*; all of these with a high confidence and leaving some low abundance taxa out of the analysis. *Actinobacteria* is the phylum in which the difference between triatomine species can be seen more notably, especially in the case of *Ps. arthuri*, nonetheless there is variation in the four phyla (Fig. 3) The overall Margalef richness index was 0.8167, although the value varied for each triatomine species and state of infection (Fig. S2).

The composition of bacterial phyla in the gut of the insect was significantly different between triatomine species ($x^2 = 133130$, df = 1134, $P < 0.0001$) (Fig. 2A, Fig.S2), while there was no significant difference between states of *T. cruzi* infection (Fig. 2B). CIRCOS plots showed that *T. cruzi* positive samples presented a higher association with *Actinobacteria* (40%, approximately) and *Proteobacteria* (approximately 30%), while the association of *Bacteroidetes* (less than 10%) and *Firmicutes* (approximately 20%) appeared to be less clear when *T. cruzi* was present (Fig. 4A). Additionally, *Actinobacteria* seemed to be more abundant in *Rhodnius* triatomines, *Bacteroidetes* and *Firmicute*s were slightly more abundant in *Ps. arthuri*, and *Proteobacteria* was more abundant in *Triatoma* and *P. geniculatus* (Fig. 4B). Putting both variables together (triatomine species and infection state),

it can be observed that the *P. geniculatus* samples associated with *Proteobacteria* are negative for *T. cruzi* and the same happens between *Firmicutes* and *Ps. arthuri*. Despite the latter being the most evident ones, there are differences in the abundance of all the phyla between *T. cruzi* positive and negative samples (Fig. 4C).

Abundance of bacterial phyla was also compared between DTUs, and *Actinobacteria* had its highest abundance on TcII-TcVI samples (58.35%), while *Proteobacteria* was the most abundant phylum in TcI samples (35.52%). *Firmicutes* abundance remains constant independently of the DTU, but the rest of the structure of the bacteriome changes in dependence of this variable (Fig. S2).

Feeding sources identification

A total of 21 feeding sources were detected in the 55 insects collected as a result of the analyses of an approximate total of 6 million reads and a Sanger sequencing procedure (Fig. 5A). These feeding sources were composed by human (*Homo sapiens*), non-human primates (*Alouatta carata* and *Xenothrix mcgregori*), mole (*Talpa occidentalis*), opossum (*Didelphis marsupialis* and *Monodelphis domestica*), anteater (*Myrmecophaga tridactyla*), wild boar (*Sus scrofa*), rodent (*Ochotona koslowi* and *Rattus norvegicus*), sheep (*Ovis aries*, *O. canadensis* and *Capra aegagrus*), horse (*Equus caballus*), rabbit (*Oryctolagus cuniculus*), cat (*Felis catus*), dog (*Canis lupus familiaris*), and reptiles (*Laudakia tuberculata*, *Varanus flavescens* and *Daboia russelii*). The predominant feeding source found was *Homo sapiens* (53.86% of the reads), followed by *Equus caballus* (21.71%) and *Coendou melanurus* (10.34%) (Fig. 4B). The other feeding sources found had < 10% of presence, being the least abundant *Daboia russelii* (string viper) (0.01%), *Myrmecophaga tridactyla* (giant anteater) (0.02%), and *Talpa occidentalis* (iberian mole) (0.03%). The information regarding Sanger

sequencing can be found in the Supplementary information (Table. S3). The feeding behaviour of insects was different between triatomine species ($x^2 = 6486400$, df = 533, $P < 0.0001$) but there was not a statistically significant difference between the feeding behaviour of triatomines that were positive and negative for *T. cruzi*, despite showing certain differences (Fig. 5C, 5D). A trend can be seen where the only feeding source that appears to be equally fed from is *Homo sapiens*, while for the other vertebrates a preference is observed: *T. maculata* appears to prefer domestic vertebrates than sylvatic ones, like *P. geniculatus*, while *R. prolixus* is the most generalist triatomine species.

The CIRCOS plot that compared triatomine species and vertebrate feeding sources, revealed that feeding preferences vary depending on the triatomine species (Fig. 6). *Rhodnius* triatomines and *Felidae* showed an association (approximately 20% of the samples showed this association), while *P. geniculatus* and *Canidae* did as well (more than 20% of the samples) and *Ps. arthuri* had an association with *Didelphidae* vertebrates (almost 20% of the samples). *Triatoma* is more homogeneous in its feeding preferences, showing a slightly higher preference for *Canidae* in 20% of the samples used (Fig. 7).

Diversity analysis

For the alpha diversity analysis, we calculated Inverse-Simpson diversity index for bacteriome data separated by triatomine species and *T. cruzi* infection. For the bacteriome data, we found that the Inverse Simpson index had its highest and lowest values in *R. prolixus* and *Ps. arthuri*, respectively (Fig. 7A). For the case of *T. cruzi* infection, Inverse Simpson index was predominantly higher in negative samples (Negative average = 1.010×10^{-4}; Positive average = 7.628×10^{-5}) (Fig. 7B).

PCA was performed for the beta diversity analysis and it resulted in two clusters: one showing all the 55 individuals evaluated and one showing all the variables considered in the study (bacteriome and feeding sources together). This analysis showed a single defined group in the plot containing individuals, and this group corresponded to *Ps. arthuri*, which is highlighted in the plot made (Fig. 7C). *Triatoma, Rhodnius* and *P. geniculatus* individuals were dispersed and therefore did not form any group. In the plot made with variables, a big part of the bacteriome components and feeding sources are positively correlated and have the biggest contributions to the PCA coordinates (Fig. 7D). It can be seen here that the triatomines that fed on *Viperidae, Erethizontidae* and *Platyrrhini* have a similar bacteriome structure. These groups are positively correlated with *Elusimicrobia, Planctomycetes, Nitrospirae, Acidobacteria, Chloroflexi* and *Chlamydiae*, therefore these bacteria should be abundant in the triatomines that feed on the vertebrates just mentioned. On the other hand, there are some bacterial phyla negatively correlated with certain feeding sources. Bacteria like *Bacteroidetes, Tenericutes, Spirochaetes, Synergistetes, Fusobacteria* and *Chlorobi* have a negative association with *Canidae, Didelphidae, Myrmercophagidae, Hominidae, Bovidae* and *Talpidae.*

Discussion

The understanding of the microbiota present in vectors of human-borne diseases is pivotal for the comprehension of the relationships with the parasites they transmit. To our knowledge, this is the first study that has used next generation sequencing (NGS) technologies in several *T. cruzi* vector species to depict their gut bacteriome and feeding sources. Our results suggest that NGS technologies can be used to detect a big part of the diversity harboured in insect guts and, additionally, to identify the different feeding sources

that triatomines have fed on. Therefore, these technologies can have a crucial role in the understanding of the ecology of Chagas disease, especially the transmission dynamics of the parasite.

Regarding the bacteriome information, we found 27 bacterial phyla (Fig. 2), which accounts for a higher diversity than reported before for triatomines [18, 19, 23, 44] and can be a consequence of the methodology herein used, which had more sensitivity (higher number of reads) and used different primers (a more informative region in the case of 16S gene fragment), characteristics that can condition the results [45]. Nonetheless, other studies have found four predominant phyla in the gut of triatomines: *Actinobacteria, Bacteroidetes*, *Firmicutes* and *Proteobacteria* [18, 19, 30], that we found as well in our study. There were no significant differences between positive and negative samples for *T. cruzi*, but each species showed a different bacteriome signature (Fig. 2, Fig. 3), which confirms that bacterial abundance is species-dependent as previous studies have suggested [20]. Despite the lack of statistical difference, insects with no presence of *T. cruzi* had a higher bacterial diversity (Fig. 7). In previous studies, bacterial diversity tended to increase with *T. cruzi* infection [21, 22], which was proposed as a defense mechanism of parasite immune modulation [20]. Nonetheless, some studies have found that *T. cruzi* immune response modulation decreases gut microbiota given that this can trigger antibacterial activity, which is a possible explanation for the decrease of bacterial diversity in the *T. cruzi* positive samples in our study [9, 46]. Additionally, the samples used in this study were field-collected, unlike a big part of the previous studies performed [18, 19] and this can also contribute to the explanation of the difference in the apparent effect of *T. cruzi* in the bacteriome diversity as previously reported [47].

Given that CIRCOS plots did not show a difference between *T. cruzi* positive and negative states, especially in the case of the four predominant phyla, we can suggest that these bacteria are present in a manner that is independent of the parasite, therefore, the parasite is not affecting their density in the gut. If the parasite does not affect some bacteria in the insect gut, this might mean that only certain bacteria are involved in the immune response of the insect when *T. cruzi* is detected. This can translate in only some bacteria possessing mechanisms to recognize *T. cruzi* upon its entrance and, consequently, reacting for the immune response to act, which has also been suggested previously [48]. In the future, this could be addressed performing a mechanistic experiment in which the functionality of the bacteria is considered as the peak of the study.

Despite having a statistically significance, there are common trends between the bacteriome of different triatomine species, like the presence of four predominant phyla (Fig. S2A). *Actinobacteria* possesses a wide range of physiological and metabolic properties, such as the formation of secondary metabolites and antimicrobial bioactive compounds [49]. *Proteobacteria* is tightly related to dysbiosis in vertebrates and harbors a great part of the functional variation in contrast with other highly abundant phyla like *Bacteroidetes* or *Firmicutes* [50, 51]. *Bacteroidetes*, commonly found in the gut microbiota of animals, is known for its ability of degrading polysaccharide due to its numerous carbohydrate-active enzymes[52, 53]. Lastly, *Firmicutes* has several genera that may help in the salvage of energy from unabsorbed dietary carbohydrate given their carbohydrate fermenter abilities [54]. Additionally, *Actinobacteria* and *Firmicutes* are considered to supplement nutrition and are required for normal growth in insects [45, 55, 56]. Some bacteria belonging to *Bacteroidetes*, can degrade porphyrans, and polysaccharides, enabling degradation of food components in

the insect [57]. Lastly, *Proteobacteria* can be important in the digestion, immunity and reproduction of the insect [56, 58–61]. Interestingly, some *Proteobacteria* (that were not found in this study) can be important in the early development of some insects, even causing a trypanosome-refractory phenotype in adult tsetse flies (*Glossina spp.*) [59]. In our study, these bacteria were the ones with the highest abundances, and is probably due to the assistance they provide to the physiological processes just mentioned, which can easily make them fundamental in the system of the insect and the vertebrates it feeds on.

Regarding the bacterial genera, apparently *Proteobacteria* is the phylum in which more variation between species can be found (Fig. 3). *Wolbachia* was only found in *R. prolixus*, while in previous studies it has been found in *R. pallescens* and *R. prolixus* [18, 19, 62]. Given that these bacteria can alter the reproductive tissue of its host, they have been suggested as a tool for vectorial control [62] and this can be the reason why we found it only in one triatomine species and with one of the lowest abundances detected. There are also differences between previous studies and this one regarding the presence of some bacteria in certain triatomine species [21, 30]. These differences suggest that the presence of certain bacteria can depend on variables that have not been carefully considered yet and, therefore, more studies need to be performed. Additionally, there were some individual results that are worth noting and more studies may be necessary to fully understand them. For example, we found *Cyanobacteria* in triatomine guts. These bacteria are not considered as related with the bacteriome of triatomines or any other insect [45, 47] but similar bacteria have been found in the human gut bacteriome [63], therefore their provenance is worth studying. We also found *Tenericutes* in the triatomine gut and, given that this type of bacteria can be commensals or parasites towards animals [64], its presence can potentially be related to the

feeding sources of the triatomines, but we cannot assure this by using the information we have.

Given that the bacteriome composition seems to be dependent on the triatomine species, differences between the digestive tracts of triatomine species can be a determinant factor. According to the few studies existent, not much is known about the physiology of the digestive tract in triatomines given that most of the studies have been made in *R. prolixus* [65]. Nonetheless, it is known that *Triatoma* has several similarities with the *Rhodnius* gut [65–67], given that genes related to basic functions were abundantly transcribed and the immunity related transcripts are almost the same between these species [65] ; and in the case of *Panstrongylus* studies have focused on the salivary glands [68–70]. Even though, the currently available information points to the possible lack of differences between the digestive tract of triatomine species, lots of studies are required to confirm this. Again, the diet preferences can have an important role here given that the structure of cells can vary between vertebrates [71] and, evolutionarily, this could shape the requirements for the gut of each triatomine species. An additional important factor here is the saliva of the insect, given that its effect can be different depending on the triatomine species [72] and this can condition the posterior interaction of the insect gut with the blood meal. Therefore, it is important that studies considering this characteristic are developed in the future. Lastly, there can be trypanolytic factors in the triatomine gut that can also condition the development of the parasite in the insect and might have an interaction with bacterial communities [5]. Therefore, several aspects of the digestive tract (physiology, anatomy and interactions) remain unknown to depict plausible explanations regarding unique bacteriome signatures per triatomine species.

Regarding the feeding source information, we found 21 mitochondrial reads from vertebrates (Fig. 5), which is higher than what previous reports show [23, 27]. We also found more than one vertebrate feeding source per triatomine (Fig. S4), which confirms what has been shown before about the possibility of a constant feeding source change in an individual [23]. The fact that our study showed more feeding sources than the previous one can be possible since we evaluated other and multiple species and, additionally, the number of samples used by us was higher. The predominant feeding source detected was *Homo sapiens* (Fig. 5) and more than half of our evaluated samples were infected with *T. cruzi*, just like it has been detected in previous studies made with *Triatoma* [22, 73]. Additionally, a considerable number of samples in our study had some characteristics linking them to domestic ecotopes (Table S1), which highlights even more the risk people have to acquire *T. cruzi* in their own homes.

Despite having a statistical difference, one can identify some trends in triatomine species respecting feeding sources (Fig. 5, Fig. S4A). The reason why *T. maculata* and *P. geniculatus* appeared to prefer domestic vertebrates is possibly related with their frequent presence in domestic ecotopes [15, 74, 75]. A possible explanation for the high abundance of *Homo sapiens* is the presence of human settlements even in sylvatic ecotopes all over the country. Because of this, humans could be a usual component in the diet of triatomines, even more than other vertebrates. Additionally, it has already been reported certain preference of triatomines for *H. sapiens* blood [76], which could also contribute to the number of reads found for *H. sapiens* in this study. The fact that almost all the individuals that fed from certain feeding sources were also positive for *T. cruzi*, while there are individual that feed from other vertebrates and are negative for *T. cruzi* (Fig. S4B) can be important in the understanding of

the ecoepidemiology of the Chagas disease, given that it adds information to what is known about the vertebrates involved in the transmission cycles, for both domestic and sylvatic ecotopes. Nonetheless, there can be missing information in what we found, given the reduced number of samples, the number of departments of provenance of the triatomines we used, and the methodology we used. Additionally, this methodology can be influenced by the time passed since the last time the triatomine fed on a vertebrate. There can be a relation between the time passed and the number of reads detected where the more recently the triatomine fed on a vertebrate, the more number of reads can be obtained. Therefore, not finding certain feeding source is not concluding evidence of the lack of this feeding source in the diet of the triatomine. Nonetheless, it is important to take into consideration that in some occasions, it can be hard to extract intestinal contents from insects, especially when they have been starved for a long time (which could be the case in our study given that we had no information about the diet status of the samples collected) [71]. Additionally, given the differences between the erythrocyte structure, some feeding sources tend to persist more time in the gut of the insect, which can alter the proportions detected [71].

The relationship between each bacteriome component and feeding source can be seen in the PCA analysis (Fig. 7). Additionally, domestic feeding sources are positioned in a different part of the graph than sylvatic feeding sources. This means that probably the bacteriome composition is conditioned by the ecotope to which the feeding sources belong. The same correlation between bacteriome structure and feeding source has been observed in other animal species [77–79]. Despite ecotope might be conditioning the bacteriome structure, there were not compelling differences between triatomines given that they feed on sylvatic and domestic feeding sources simultaneously. This might be proof that triatomines

constantly switch ecotopes and further studies should be made to prove this, given that it can be an important aspect in the understanding of the triatomine ecology and, ultimately, *T. cruzi* ecology. Additionally, it is known that diet has been a shaping force in the evolution of the *Triatominae* subfamily, changing even the conditions for reproduction and the patterns of population density [80, 81]. Due to this link of *Triatominae* to evolution and radiation of Neotropical mammals and birds [82], the diet composition not only affects the microbiota present in the insect but also defines the evolutionary pathway of the subfamily. In fact, the effects of the bacteriome in the immune response and general health, can be related to this as well given that diet conditions the bacteriome composition. Therefore, diet is pivotal in several aspects of triatomines.

This study does not show data regarding the other components of the microbiome of triatomines (fungi, eukaryotes and viruses). Even though, the eukaryome and virome do not seem to be described yet. Previous studies have identified fungi such as *Penicillium*, *Aspergillus* and *Acremonium* in *Triatominae* species [83] and for example in beetle species, it has been observed a trend where fungal communities change [84]. Regardless of more studies made with the olive fruit fly [85], leaf-cutting ants [86], there is no information about how fungi can alter the development of parasites or any activity related. Despite the availability of information about the mycobiome [83, 87, 88], future studies should extensively characterize fungi, viruses and eukaryotes present in the triatomine gut of each species to understand the totality of their microbiome and its possible effects on other variables related with the ecology of these insects and Chagas disease.

In conclusion, as a result of the methodology herein used, we were able to find a higher diversity than reported previously for bacterial taxa present in the triatomine gut and

triatomine feeding sources. Bacteriome structure seems to vary in a species-dependent manner, while preferences of feeding sources appear to be homogenous between the generalist triatomine species. According to our results, ecotope and geographic location might be variables that need to be studied more profoundly as sources of variation in the bacteriome structure. This study allowed to perform a first characterization of the bacteriome structure of the main triatomine genera in Colombia and more studies should be made to completely understand the ecology and role of triatomine gut microbiome interacting with *T. cruzi*; which might provide new insights regarding host-pathogen interactions and subsequently to depict the global ecology of Chagas disease.

Figure Legends

Figure 1- Geographical distribution of 56 triatomine samples used in this study. Legends differentiate each triatomine genus collected and figure size symbolizes triatomine density in the location.

Figure 2- Relative abundance of bacterial phyla in each triatomine individual tested separated by (A) species, and (B) *T. cruzi* infection.

Figure 3- Relative abundance of bacterial genera from the most abundant bacterial phyla per individual: A) *Actinobacteria*, B) *Bacteroidetes*, C) *Firmicutes*, and D) *Proteobacteria*.

Figure 4- Circular webs made with CIRCOS online tool representing the relative abundance of the bacterial phyla detected in A) triatomine samples positive and negative for *T. cruzi* infection, B) each triatomine species, and C) triatomine samples positive and negative for *T. cruzi* infection in each triatomine species.

Figure 5- A) Relative abundance of the detected feeding sources in each triatomine sample. B) Number of reads obtained for each vertebrate. Bar colors represent the triatomine species in which these reads were found. C) Relative abundance of the detected feeding sources in triatomine samples positive and negative for *T. cruzi* infection. D) Circular web made with CIRCOS online tool representing the relative abundance of the feeding sources detected in *T. cruzi* positive (blue) and negative (orange) triatomine samples.

Figure 6- Circular web made with CIRCOS online tool representing the relative abundance of the feeding sources detected in each triatomine species (or genus) evaluated. Feeding sources are conveniently shown in a new arbitrary grouping.

Figure 7- Alpha (A and B) and beta (C and D) diversity analysis. Inverse Simpson index for A) each triatomine species and B) *T. cruzi* infection. Principal coordinate analysis (PCoA) shown C) per individuals, and D) per variable. Colours indicate the quality of representation or for each variable considered or the contribution of said variable to the principal coordinates. Fig. 7C shows a violet circle in which *Ps. arthuri* samples are delimited, given that this is the only species that presented grouping in the plot.

Supporting files

Figure S1- Rarefaction curves of 16S rDNA of individuals triatomines belonging to A) *P. geniculatus*, B) *Ps. arthuri*, C) *Rhodnius* (*R. pallescens* and *R. prolixus*), and D) *Triatoma* (*T. maculata* and *T. venosa*). Continuous lines represent the *T. cruzi* positive individuals and dotted lines represent the *T. cruzi* negative samples.

Figure S2- Relative abundance of bacterial phyla in A) each triatomine species evaluated, and B) different *T. cruzi* DTUs.

Figure S3- Margalef index for bacteriome data separated by A) triatomine species, and B) *T. cruzi* infection.

Figure S4- Relative abundance of A) *T. cruzi* positive and negative triatomines for each vertebrate feeding source, and B) triatomines species for each vertebrate feeding source.

Figure S5- Vertebrate feeding sources for each triatomine species in dependence of the geographical precedence. A) *P. geniculatus*, B) *R. pallescens*, C) *R. prolixus*, and D) *Triatoma* (*T. maculata* and *T. venosa*).

Table S1- Complete dataset of the 55 triatomines used in this study with ID code, qPCR result, DTU information obtained through conventional PCR, results of the discrimination between *T. cruzi* and *T. rangeli*, triatomine species, part of the body of the triatomine used (tagma), results of conventional PCR for 12S rDNA for the samples it was used, geographical information (department and town), and complementary information of the insect (ecotope, life stage and sex).

Table S2- Scientific and common name of every species in the database constructed as a reference 12S sequences dataset.

Table S3- Triatomines with Sanger sequencing results for 12S rDNA. Complementary information like triatomine species and *T. cruzi* infection was added.

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