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CARRERA DE MICROBIOLOGÍA

Caracterización del microbiota en el intestino de *Rhodnius ecuadoriensis*, insecto transmisor de la enfermedad de Chagas en las regiones de la Costa Central y Sur Andina del Ecuador

Disertación previa a la obtención del título de Microbiólogo

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CERTIFICACIÓN

Yo Anita Gabriela Villacís Salazar PhD, certifico que la disertación de Microbiología del estudiante Juan Francisco Villacís Bolaños ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación.

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Quito, 29 de Enero de 2024

DEDICATORIA

Mamá, eres mi todo. Hoy quiero dedicarte este texto, porque tú eres la persona más importante de mi vida. Eres mi ejemplo a seguir, gracias por todo lo que has hecho por mí, por tu amor incondicional, por tu apoyo incansable y por tu guía. Gracias por enseñarme a ser fuerte, a creer en mí mismo y a seguir mis sueños. Eres la mujer más maravillosa que conozco, y estoy muy orgullosa de ser tu hijo.

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TABLA DE CONTENIDO

1 ABSTRACT 10

2 INTRODUCTION 10

3 METHODOLOGY 12

3.1 STUDY AREA 12

3.2 TRIATOMINE COLLECTION 13

3.3 LABORATORY TRIATOMINES 14

3.4 EXTRACTION OF INTESTINAL CONTENTS (I.C.) 14

3.5 DNA EXTRACTION AND PCR AMPLIFICATION 14

3.6 METAGENOMICS AMPLICON 14

3.7 BIOINFORMATIC ANALYSIS 14

4 RESULTS 15

4.1 BACTERIA GENUS COMPOSITION OF *RHODNIUS ECUADORIENSIS* WILD COLLECTED. 15

4.2 COMPARISON OF THE MICROBIOTA BACTERIA'S (GENUS OF BACTERIA) OF *R. ECUADORIENSIS* BACTERIA WILD COLLECTED VS. RAISED IN THE LABORATORY 16

4.3 DISTRIBUTION OF BACTERIAL GENERA IN ABSENCE AND PRESENCE OF *TRYPANOSOMA CRUZI.* 18

5 DISCUSSION 18

5.1 LIMITATIONS 22

6 REFERENCES 23

LISTA DE TABLAS

TABLE 1. SHANNON DIVERSITY INDEX FOR GENERA AND SPECIES 17

LISTA DE FIGURAS

FIGURE 1. COMMUNITIES SAMPLED IN ECUADOR 13

FIGURE 2. BACTERIAN'S MICROBIOTA (BACTERIA GENUS) COMPOSITION OF NYMPHS AND ADULTS OF WILD *R. ECUADORIENSIS*................................. 16

FIGURE 3. COMPARISON OF THE MICROBIOTA BACTERIA'S (GENUS OF BACTERIA) OF NYMPHS AND ADULTS OF *R. ECUADORIENSIS* WILD COLLECTED AND RAISED IN THE LABORATORY. 17

FIGURE 4. DISTRIBUTION OF BACTERIAL GENERA IN ABSENCE AND PRESENCE OF *TRYPANOSOMA CRUZI.* 18

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Characterization of the microbiota in the intestine of *Rhodnius ecuadoriensis*, an insect that transmits Chagas disease in the Central Coast and South Andean regions of Ecuador.

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1 Abstract

Chagas disease is a neglected tropical disease, endemic to Ecuador, caused by the *Trypanosoma cruzi* parasite that is transmitted mainly by the feces of infected Triatomines. In Ecuador the main vector is *Rhodnius ecuadoriensis* which is distributed in several provinces of the country. More than 40% of these insects have *T. cruzi* as part of their intestinal microbiota. For this reason, the objective of this research was to characterize the intestinal microbiota of *R. ecuadoriensis*. The methodology used for this research was based on active wild collection. The intestinal contents and subsequently the DNA were extracted from the collected insects (adults and nymphs V), as well as the insects kept alive in the CISeAL. Finally, the samples were analyzed by metagenomics extensions based on the different selected criteria. The intestinal microbiota of *Rhodnius ecuadoriensis* presents a marked divergence between insects raised in the laboratory and wild collected. This difference is observed in all stages and is similar between insects from Loja and Manabí. A large loss of microbial symbionts is observed in laboratory-bred insects.

2 Introduction

Chagas disease or American trypanosomiasis is a zoonotic disease, classified in 2005 as a neglected tropical disease (1) and is an endemic disease in 21 Latin American countries. Initially, the cases were mainly concentrated in rural areas but now, most of the reported cases, occur in urban areas, due to population movement (1). This causes the spread of the parasite in non-vectorial ways and its extension to other countries around the world. In Ecuador, as in many other countries where the disease is endemic, it is caused by *Trypanosoma cruzi* infection, which is transmitted by a strict hematophagous insect of the Triatominae subfamily, through feces or urine contaminated by the parasite (2).

The Pan American Health Organization (PAHO) (1) reports that approximately 70 million of people can contract Chagas disease, more than 6 million of people already suffer from it, and there are 30,000 new cases each year. At the national level, a seroprevalence of 0.65% was estimated in the South Andean region, 1.75% in the Amazon region, and 1.99% on the Coast Central (3).

In Latin America, more than 150 species of Triatomines have been reported (4), while in Ecuador, 16 different species of these insects have been recorded. Among them, 13 have epidemiological relevance (5,6), highlighting *Rhodnius ecuadoriensis* as the main vector of the disease (7,8). It is widely distributed in provinces such as Santo Domingo de los Tsáchilas, El Oro, Guayas, Los Ríos and Manabí, on the coast and in the temperate valleys of Loja, in the Andean Sierra, as well as in northern Peru (9–11).

The presence of *R. ecuadoriensis* in several provinces of Ecuador is of extremely importance for the public health of the country. In addition to the i) ecological alterations, ii) permanent or transitory variations, iii) disturbances of the characteristics of the ecosystem, as a consequence of internal or external actions, by human activity, they have shifted the frontier of transmission cycles. This displacement goes from being sylvatic to peridomiciliary and domiciliary (12).

The main problem is due to the fact that triatomines are obligate hematophagous in all their stages of development, that is, they need to feed on vertebrate blood to complete their cycle. Triatomines feed on various blood sources, including *T. cruzi* infected mammals, which serve as reservoirs for the disease and a form of vector propagation to humans (13).

Rhodnius ecuadoriensis is widely distributed in various environments. Its blood source from domestic animals in the coastal provinces and in the highlands of southern Ecuador up to northern Peru, added to its synanthropic strength, make it one of the main vectors of *T. cruzi* in the country (14). Their infection rates with *T. cruzi* exceed 40% (15,16). Therefore, this species is a priority for entomological surveillance.

When the *Rhodnius ecuadoriensis* feed on an infected host with *T. cruzi*, ingests trypomastigotes which become epimastigotes, which multiply and colonize strictly the midgut of the insect to finally end up as metacyclic trypomastigotes in the hindgut and spread through the insect's feces, converting to *T. cruzi* in part of the insect's gut microbiome (17–19).

The set of these microorganisms that develop and reside in symbiosis with a host is considered the microbiome; in the case of the intestinal microbiome, it consists of several members of various kingdoms such as bacteria, fungi, viruses, archaea and protozoa (20). These microbial communities are highly dynamic, they evolve throughout the life of the host and in insects a close evolutionary relationship between the microbiome-insect is known, although the real extent of the associations is mostly unknown (21). Current evidence reveals that these microorganisms act as behavior modulators, protectors against possible pathogens, support nutrition, facilitate essential compounds and more essential functions for insects than are currently known (21,22).

The microbiota can be classified by its location as “endosymbiont” for microorganisms found inside the insect body or “ecto-symbiont” for microorganisms found on the outside of the insect (21). In the same way, those microorganisms that may or may not live in association with the insect can be subclassified as: i) “facultative” or “obligate” for those that strictly need to associate with the insect to survive, ii) “commensal” for the microorganisms that obtain a benefit from the insect without causing any damage, and iii) “Parasites” are those microorganisms that get benefits from their association with the insect (21).

The digestive system of *R. ecuadoriensis* is an ecological niche for many endo-symbiont microorganisms (23). The intestinal microbiota varies according to the triatomine species (24). In addition, it is influenced by factors such as: i) the type of blood diet, ii) environmental conditions, iii) the presence of competitors such as *Trypanosoma cruzi* is capable of modulating the insect's immune response in its favor (25). In contrast, some bacterial genera are capable of competing with the parasite for the ecological niche (26). In addition, some bacteria present trypanolytic activities (25,27).

However, there are some studies on the intestinal microbiota in another species of triatomines (28,29). An in the case of *Rhodnius. ecuadoriensis* ecology studies, life cycle, feeding and defecation patterns, behavior studies, molecular and phenotype research have been conducted (9,16,30,31). However, there is no previous study on the characterization of the intestinal microbiota in *R. ecuadoriensis*, despite its importance as the main vector of Chagas disease in Ecuador. Therefore, understanding the interactions between the intestinal microbiota of *R. ecuadoriensis* and *T. cruzi* is essential for the epidemiology of the disease in the country and, in this way, to be able to propose possible strategies to interrupt the transmission cycle in endemic areas (14,32–34).

Therefore, with this study we want to answer the following questions: i) if there are characteristic bacterial genera of *Rhodnius ecuadoriensis*, ii) if there are differences between the microbiota of parasitized triatomines and those not parasitized with *T. cruzi*, iii) if there are differences between the intestinal microbiota of sylvatic triatomines versus those that were reared in the laboratory and, finally, iv) whether the intestinal microbiota of the insect inhibits the development of *T. cruzi*.

3 Methodology

3.1 Study area

This study was conducted in three rural communities with high presence of *Rhodnius ecuadoriensis* in Ecuador. Two communities, Guara (1064–1450 meters above sea level [masl]) and Bellamaría (1000–1384 masl), belongs to Calvas County in Loja province. These communities presents five vegetation zones: deciduous forest, semi-deciduous forest, lowland green forest, cloud forest, and dry forest of shrubs mountain (16). The third community was Caja Fuego (∼100 masl), Portoviejo County, in Manabí province, it has a climate between dry subtropical and humid tropical. In addition, it will cover six vegetation zones: deciduous forest, semi-deciduous forest, low mountain green forest, cloud forest, dry montane shrub forest and tropical savannah (15).

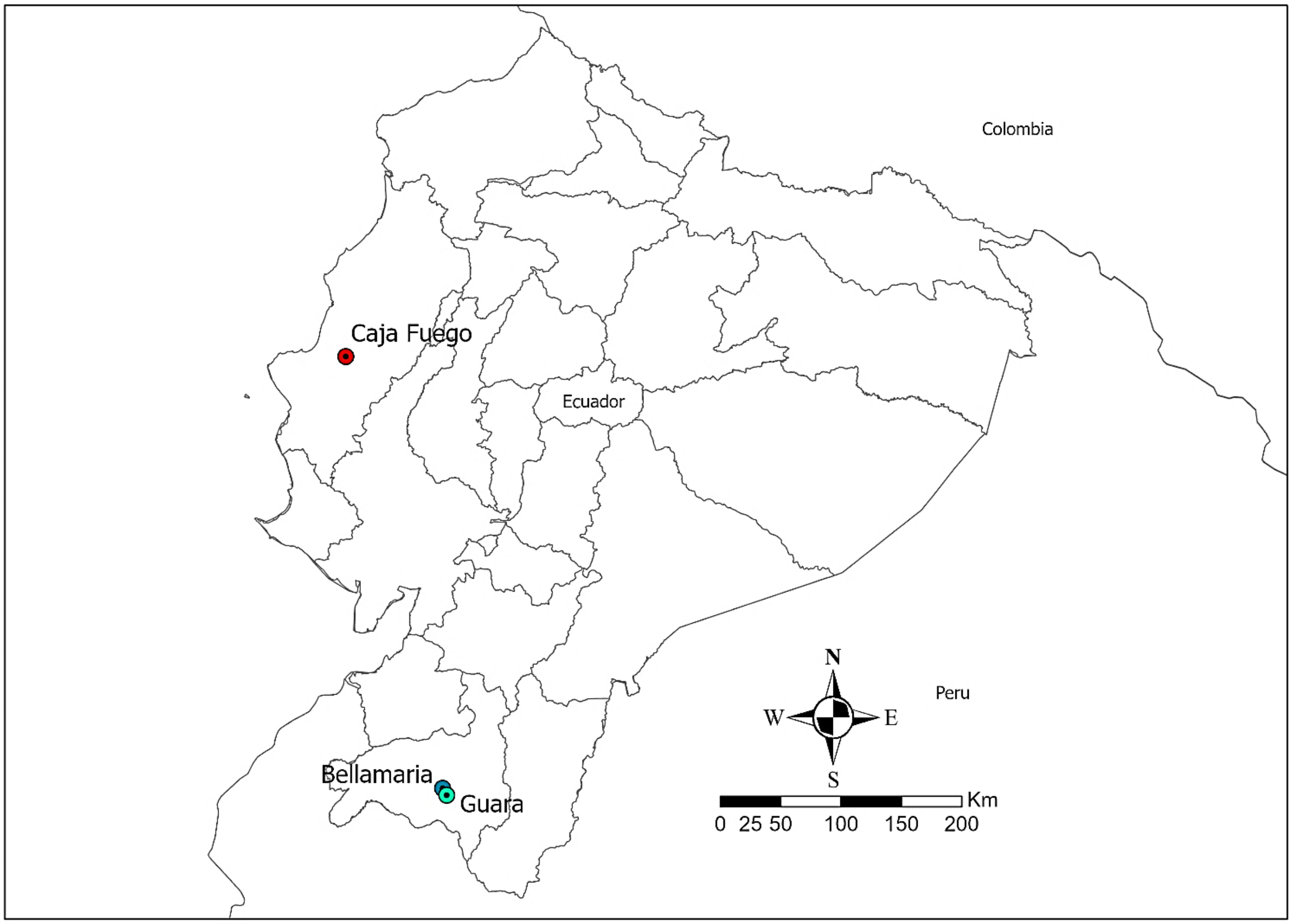

Figure 1. Map of Ecuador. Caja Fuego community, Coastal Region where Manabí Province belongs, and Bellamaría and Guara communities, southern Andean Region, where Loja Province belongs.

3.2 Triatomine collection

The collection of triatomines will be carried out with collection permits 002-17IC-FAU-DNBAPVS/MA, 010-IC-FAU-DNBAPVS/MA and MAAE-DBI-CM-2021-0185, in the coastal province of Manabí and in the Andean province of Loja in Ecuador respectively, where it has been reported the presence of *R. ecuadoriensis*. During the day, domiciles and peridomiciles were searched for triatomine using the one-man-hour method as previously described in Grijalva et al. (12) and conducted by two-person skilled teams (30 min in domestic habitats and the 30 min in peridomestic habitats) from the national vector control programs under the supervision of project personnel. While, in the sylvatic habitats the searches were conducted in mammal and bird nests located in trees, bushes and piles of rocks as indicated by Grijalva and Villacís (14) and Villacís et al. (30). Live and dead insects were collected, and then were transported by the mobilization permits number MAAE-CMARG-2020-0178 from Loja and Manabí in sterile vials to the Center for Research for Health in Latin America (CISeAL). Details of place of capture (domicile, peridomicile or sylvatic species, number of insects found dead or alive, and insects' developmental stages and sex (of adults) were performed using a dichotomous key by Lent and Wygodzinsky (35).

3.3 Laboratory triatomines

The insects were maintained under controlled conditions of humidity, temperature and photoperiod described by Villacís et al. (31) and the insects were periodically fed with human blood meal every 15 days with Hemotek membrane feeding system (Hemotek Ltd., Blackburn, UK) for 45 minutes (36,37).

3.4 Extraction of Intestinal Contents (I.C.)

Each insect was placed in a sterile tube at -20ºC for 20 min, rinsed with 70% ethanol and sterile distilled water to remove dirt and accompanying microbiota (38). Immediately, the intestinal contents would be extracted under aseptic conditions in a laminar flow chamber, a transverse cut was made at the genitalia tip with a sterile scalpel. With the aid of a variable volume micropipette and sterile filter tips, hindgut and feces were removed.

3.5 DNA extraction and PCR amplification

DNA extraction was performed using the ZymoBIOMICS DNA Microprep Kit based on the protocol established by the manufacturer with the following variations: The intestinal content was recovered directly in the lysis solution and 10ul of proteinase K was added, with incubation at 55ºC for 50 min at 300 rpm. DNA concentration was measured using Nanodrop 2000 (ThermoScientific). The Bacterial 16S DNA was amplified with the primers F27 and R1492, used the GoTaq Flexi DNA Polymerase kit and dNTP Mix of the Promega. Amplification starts with 5 min of initial denaturation at 95ºC, 35 cycles of (1 min of denaturation at 95ºC, 1 min of hybridization at 59ºC, 1 min elongation at 72ºC) and 10 min final elongation at 72ºC according to (39). Similarly, for the amplification of DNA of *Trypanosoma cruzi* were used the primers TcZ1 and TcZ2 with 3 min of initial denaturation at 95ºC, 40 cycles of (20 sec of denaturation at 95ºC, 15 sec of hybridization at 59ºC, 30 sec elongation at 72ºC) and 7 min final elongation at 72ºC (40). Finally, a 1.5% agarose electrophoresis was performed to confirm the presence of DNA.

3.6 Metagenomics Amplicon

Amplicon sequencing was performed using extracted gDNA previously quantifed by Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, EE. UU). From each triatomine pool, libraries were constructed with primers targeting the hypervariable regions V3–V4 of the 16S ribosomal DNA. Primer pairs used were 341F: (5′-CCTACGGGNGGCWGCAG-3′) and 805R: (5′-GACTACHVGGGTATCTAATCC-3′). Amplicons were sequenced on Illumina MiSeq platform by Biosequence S.A.S, located in the city of Quito, Ecuador.

3.7 Bioinformatic analysis

Using the Fastq files of each pool, a quality and filtering process was carried out to guarantee the taxonomic classification. For the taxonomic classification, an implementation of a high-performance algorithm of the Ribosomal Database Project (RDP) classifier described by Wang Q (41) was used. The database used is RefSeq RDP 16S v3, based on a set of FASTA files from:

https://benjjneb.github.io/dada2/training. 16S rRNA gene sequences with DADA2 format for bacteria and archaea (Version 2). After this, the analysis of the Shannon diversity index for genera and species was conducted with the following formula $H' = -\sum_i^S p_i \ln(p_i)$.

4 Results

The intestinal content of *Rhodnius ecuadoriensis* inoculated in non-differential culture media to demonstrate the presence of bacteria, obtaining little bacterial growth after 48 hours at 28±3ºC only in blood agar (Not shown). The PCR results visualized by electrophoresis of all wild insects (n=45) to detect *T cruzi* showed that 41.66% of the insects collected in Loja and 25.57% of the insects collected in Manabi were infected with *T. cruzi* as part of their intestinal microbiota (Not shown).

4.1 Bacteria genus composition of *Rhodnius ecuadoriensis* wild collected.

A total of 45 *Rhodnius ecuadoriensis* collected at the wild and grouped in 6 pools according to their stage and location included in this study, on average the predominant bacterial genera in the intestines were *Corynebacterium* 20%, *Eikenella* 14.09%, *Rhodococcus* 11.65%, *Williamsia* 5.80%, *Enterococcus* 3.95. %, *Staphylococcus* 2.53% and *Yokenella* 2.39%. (The "Other" category in this graph is the sum of all Genera with less than 1% abundance). The main species identified in the intestines were *Corynebacterium glycinophilum* 23.66%, *Eikenella corrodens* 20.14%, *Williamsia serinedens* 8.39%, *Enterococcus faecalis* 5.68%, *Corynebacterium terpenotabidum* 4.02%, *Rhodococcus marinenascens* 3.90%, *Staphylococcus xylosus* 2.35 %, *Snodgrassella alvi* 1.34% (Figure 2).

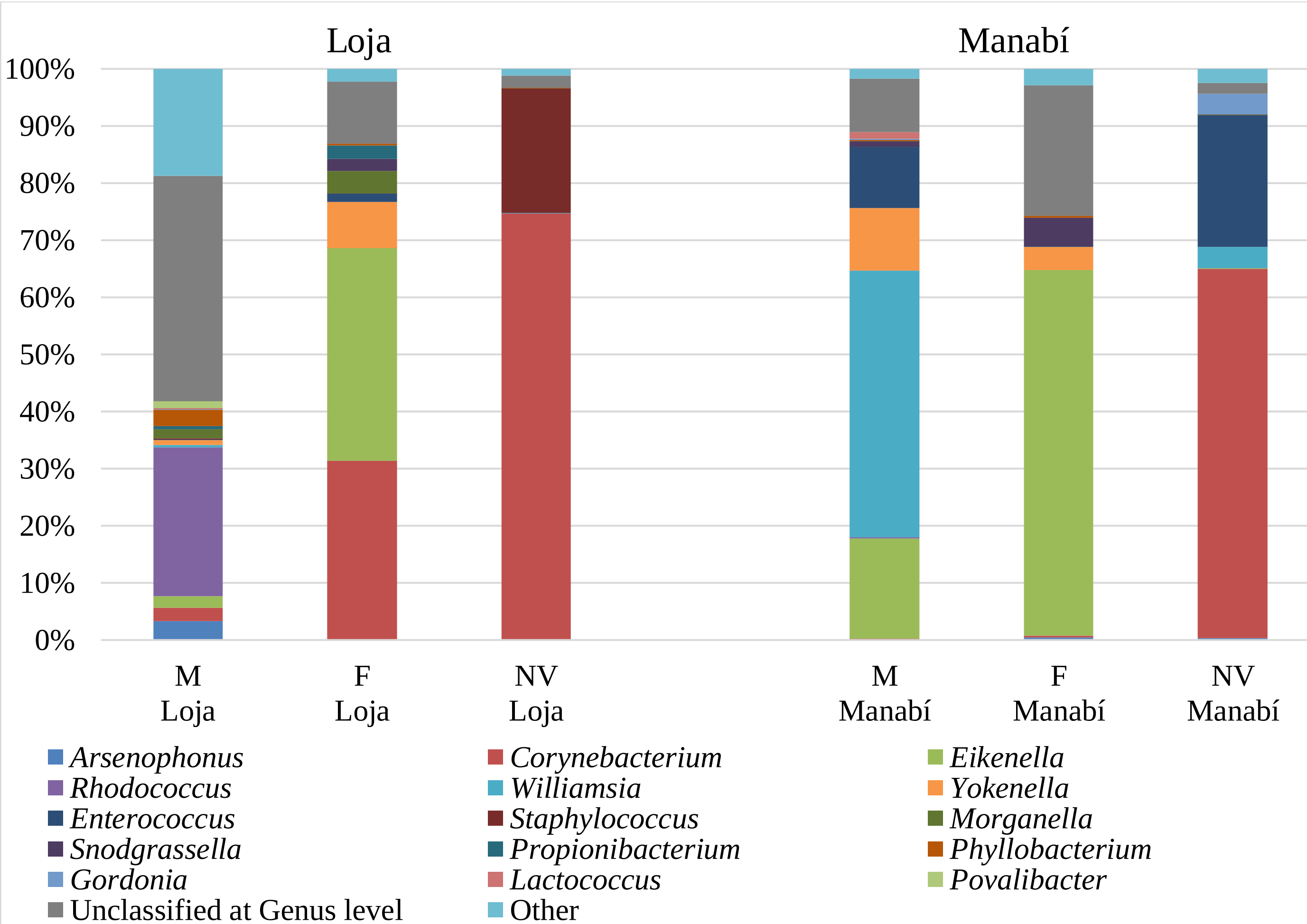

Figure 2. Bacterian's microbiota (bacteria genus) composition of nymphs and adults of wild *R. ecuadoriensis*.

*Stage: Nymph V (NV), Females (F), Males (M)

4.2 Comparison of the microbiota Bacteria's (genus of bacteria) of *R. ecuadoriensis* bacteria wild collected vs. raised in the laboratory.

Comparison of the intestinal microbiota *R. ecuadoriensis* bacteria wild collected (6 pools) and those raised in the laboratory (6 pools). The groups of insects wild collected present a greater diversity of genera, on average the most abundant bacterial genera were *Corynebacterium* 20%, *Eikenella* 14.09%, *Rhodococcus* 11.65%, *Williamsia* 5.80%, *Enterococcus* 3.95. %, *Staphylococcus* 2.53% and *Yokenella* 2.39%. The composition of the intestinal microbiota is considerably different in insects raised in the laboratory, on average the most abundant genera were *Arsenophonus* 89.07% and *Corynebacterium* 3.14%. (The "Other" category in this graph is the sum of all Genus with less than 1% abundance)

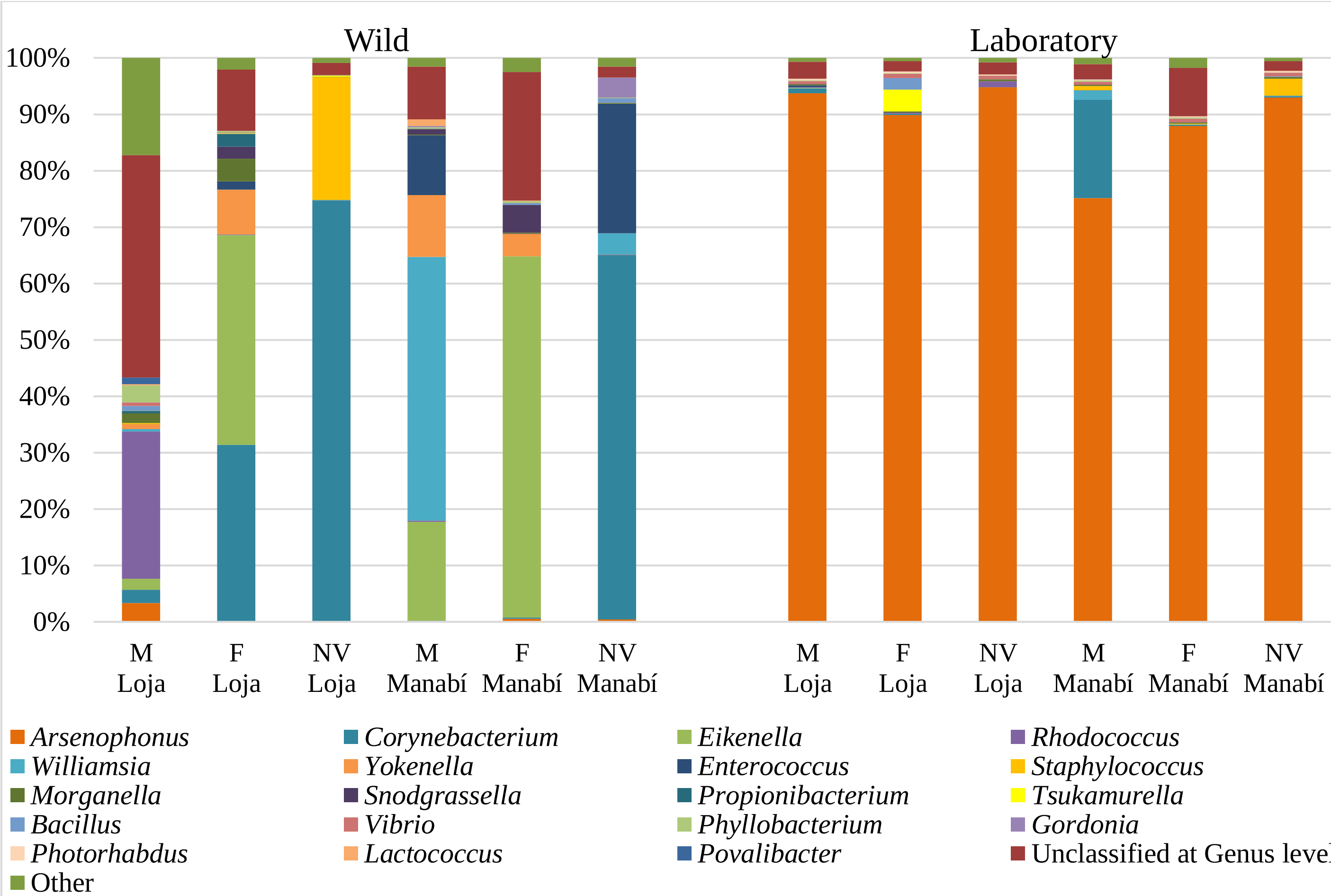

Figure 3. Comparison of the microbiota Bacteria's (genus of bacteria) of nymphs and adults of *R. ecuadoriensis* wild collected and raised in the laboratory.

*Stage: Nymph V (NV), Females (F), Males (M)

Table 1. Shannon diversity index for Genera and Species

The table shows the Shannon index of the bacterial genera and species present in wild *Rhodnius ecuadoriensis* and raised in the laboratory. The wild insects, in general, have a moderate diversity of genera and species, with an index greater than 1. However, the nymphs V (NV) from Loja have a low diversity of genera, with an index less than 1. Likewise, the female (F) from Manabi have a low diversity of species, with an index less than 1. On the other hand, the insects raised in the laboratory for both, Loja and Manabí have low or very low diversity, with indices less than 1 and 0.5 respectively.

	Wild						**Laboratory**					
	Genera			**Species**			**Genera**			**Species**		
	M	**F**	**NV**	**M**	**F**	**NV**	**M**	**F**	**NV**	**M**	**F**	**NV**
Loja	1.49	1.68	0.76	1.23	1.48	0.98	0.36	0.53	0.32	0.31	0.42	0.27
Manabí	1.66	1.18	1.15	1.4	0.94	1.61	0.9	0.56	0.39	0.83	0.46	0.36

*Stage: Nymph V (NV), Females (F), Males (M)

4.3 Distribution of bacterial genera in absence and presence of the parasite, *Trypanosoma cruzi.*

Comparison of the intestinal microbiota of *R. ecuadoriensis* with *T. cruzi* as part of its microbiota, on average the genera *Corynebacterium* 32.85%, *Williamsia* 15.59, *Eikenella* 7.69%, *Enterococcus* 5.01%, *Yokenella* 3.63% and *Povalibacter* 1.23% were the most abundant. Insects that did not have *T. cruzi* as part of their microbiota had the genera *Eikenella* 29.37%, *Rhodococcus* 12.99, *Corynebacterium* 11.18%, *Yokenella* 5.12%, *Enterococcus* 3.91% *Morganella* 2.38% *Snodgrasella* 1.89%, *Phyllobacterium* 1.69% and *Propionibacterium* 1.43% as the most abundant genera. (The "Other" category in this graph is the sum of all Genus with less than 1% abundance).

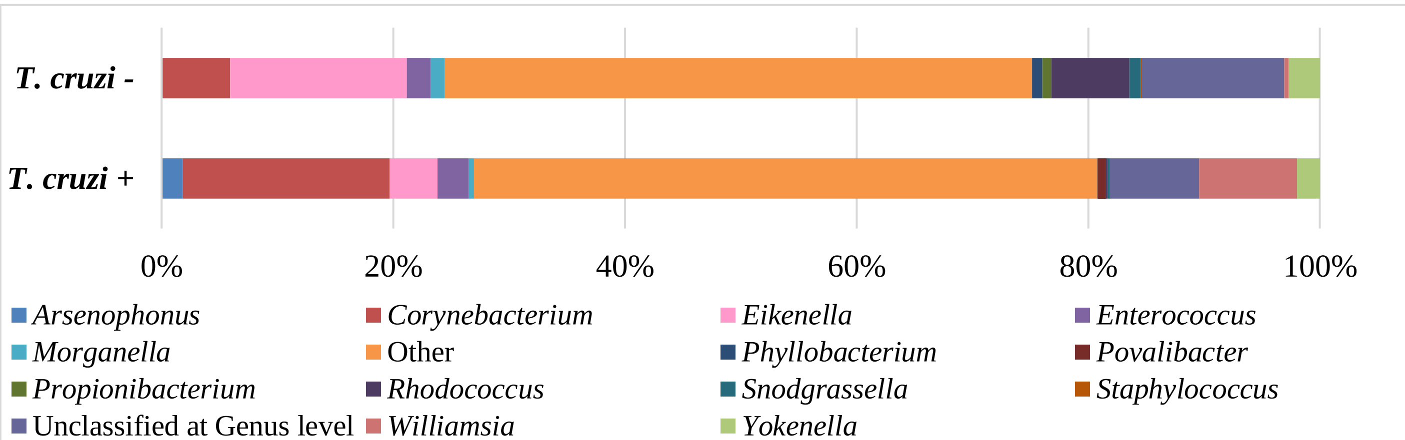

Figure 4. Distribution of bacterial genera in absence and presence of *Trypanosoma cruzi.*

5 Discussion

We present a pioneer study of the bacterial intestinal microbiota characterization using pools for extension metagenomics that allows the analysis of a greater number of sequences of *Rhodnius ecuadoriensis* in less time and low cost. In comparation with traditional methodology and isolation in culture media, which present various biases such as limited number of culturable bacteria, the specific nutritional requirements and conditions necessary for their growth in the laboratory. In this way, a greater panorama of the intestinal bacterial symbionts of *Rhodnius ecuadoriensis* was obtained, providing valuable information of the biology of the insect and the vector transmission of *Trypanosoma cruzi*.

The different insect groups (stages, habitats and province) have a unique bacterial microbiota, with a combination and bacterial diversity that are not found in other insects, the representative genera of this study being *Arsenophonus*, *Corynebacterium*, *Eikenella*, *Rhodoccoccus*, *Williamsia*, *Yokenella*, *Enterococcus* and *Staphylococcus*, which represent more than 60% of the bacterial microbiota of *R. ecuadoriensis*. In this study he results showed a differentiation between bacterial genera in the different stages of wild insects in accordance with Muñoz, et al. (42) this confirm that the digestive system of *Rhodnius ecuadoriensis* is a dynamic microhabitat and its microbiota is directly related with the i) insect stage, ii) the environmental conditions where they come from, where it develops and the presence of certain microbial groups based on what was mentioned by Guarneri & Schaub (43); Gurung et al. (20); Dumontiel, et al. (44); Lewis & Lizé (45).

If we compare between the wild insects with the insect raised in the laboratory, the results indicate an evident divergence between the bacterial communities with a predominance of the *Arsenophonus* genus with an average of 97.76% in Males, 89.83% in Females and 94.76% in NV from Loja. Likewise 75.14% in Males, 87.90% in Females and 93.02% in NV from Manabí, in contrast, their wild counterparts do not exceed 10% in accordance with what Gupta & Nair (21) proposes. We suggest that there are several ways in which these insects can acquire their intestinal microbiota, starting with hatching, contact with environmental microorganisms, hemophagy, coprophagy, cannibalism as mentioned by Guarneri & Schaub (43); Márcia et al. (46). It is presumed that the main reasons for the loss of diversity are i) due to the lack of contact with microorganisms, other than their own and those of their congeners, ii) the controlled environmental conditions, ii) the type of food and the way in which they feed and, iv) a depression of the insect's immune system is suspected to be the causes of the low diversity of bacteria in the intestine of insects raised in the laboratory. That is the reason that only wild insects provide real information on the bacterial intestinal microbiota of *R. ecuadoriensis.*

On the other hand, contrary to what was expected, *Wolbachia* genus could not be identified in any sample analyzed. *Wolbachia* is one of the most studied bacterial genera and widely distributed in different insects. It is known that it can modify the behavior of the insect in its environment, convenience to favor its transmission (45). In contrast, all groups have the *Arsenophonus* genus, which has mechanisms similar to *Wolbachia* to favor its transmission, as indicated by Lewis & Lizé (45). The presence in all insects of *Arsenophonus* confirms that it is an endosymbiont genus that is transmitted by a mixed route (47), suggesting that the high colonization rate in laboratory insects may be due to prolonged, repetitive exposure and lack of contact with other competing microorganisms, which would explain their wide abundance in reared insects in the laboratory (45).

As proposed by Ravi Durvasula et al. (48) , *Corynebacterium* is a symbiotic bacterium of triatomines, it supplies pantothenic acid to the nymphs and is necessary for the development and maturation of the triatomines, which agrees with its greater presence in wild NV nymphs. On the other hand, is very interesting find the presence of the genus *Xenorhabdus* in all the samples analyzed due to this bacterium is a symbiont of nematodes of the family Steinernematidae (Rhabditida), which are known as obligate endopathogens of insects, as indicated by Ruiz Laparra, (49). Similarly, the genus *Lactococcus* is only present in adult insects, both males and females, on the contrary, the genus *Nocardia* is only found in NV nymphs.

The genus *Rhodococcus* was found in 14 of 16 pools of samples in various proportions, confirming that *Rhodococcus* is a symbiont of *R. ecuadoriensis* as proposed by Rodríquez et al. (50). The same way, a relevant species *Rhodococcus rhodnii* symbiont of *Rhodnius prolixus* was found in 5 groups analyzed, it has been reported that this transformed bacteria produces cecropin A, which greatly reduces the parasite load of *T. cruzi* in the insect and supplies the insect with vitamin B (51,52).

In addition to this, the results show the presence of the genera *Citrobacter*, *Klepsiella* and *Pantoea*, which are nitrogen-fixing bacteria, as mentioned by Gurung et al. (20), which supply the host insects with this element. On the other hand, the results indicate that the species *Serratia marcescens* is present in 6 groups of samples, reported as a symbiont of hematophagous insects by Vieira et al. (53), some strains intrinsically possess trypanolytic activity as indicated by Rodríguez-Ruano et al. (17) giving light to future research routes to control the transmission of *T. cruzi*.

Rodríguez-Ruano et al. (17) describes *Arsenophonus triatominarum* as a bacterial symbiont in other triatomine species; this bacterium is closely related to *Triatoma infestans*. On the other hand, in the samples analyzed, found in all samples the presence of *Arsenophonus nasoniae*, which raises an interesting question: ¿Is the species *Arsenophonus nasoniae* a natural symbiont of *Rhodnius ecuadoriensis*?

The information presented is a general overview of the bacterial intestinal microbiota of the insect, the other microbial kingdoms and the interactions between the different kingdoms and the insect must be taken into consideration. Guarneri & Schaub, (43), report the presence of various fungi in the intestine of triatomines, this research did not focus on the characterization of these microorganisms, but their importance is part of the microbiota of *Rhodnius ecuadoriensis*, so the roles that they play as symbionts of *R. ecuadoriensis* could be considered (20) for future research.

Likewise, the intestinal microbiota of insects is not made up only of bacteria and fungi; other biological entities that are equally or more abundant are the viruses (54–56). In this case bacteriophages (viruses that infect bacteria), are known that these have a direct impact on bacterial populations which implies direct consequences on their host; the role of interactions between phages and insects is still unknown (57,58).

On the other hand, the literature indicates that Triatoma virus is the only virus that affects triatomines so far, due to i) its high specificity, ii) easy transmission, iii) high pathogenicity in triatomines, iv) its inability to infect other vertebrates, which, makes it a good candidate for the biological control of triatomines as indicated by Susevich et al. (56); Valles et al., (57). However, the tests carried out by Marti et al. (58) on *Rhodnius ecuadoriensis* indicate that it does not affect this species, research focused on this biological entity can lead to the possibility of modifying it and being able to make it selective for more species of triatomines.

The microorganisms found in the insect's exoskeleton will not be the same as those found inside the intestines. Likewise, it will vary according to the different intestinal structures; a clear example is the development of *Trypanosoma cruzi* that passes through various tissues/organs of the insect, where it gradually evolves while taking coming into contact with the normal microbiota of the insect, with the one that competes for nutrients and the opportunity to implant (59–61).

Insects have an immune system which gives them protection against pathogens and keeps their symbionts in balance. Naturally, the insect has resistance against the parasite as indicated by Mwangi et al. (62); Abreu et al. (63); Weiss et al. (61) but in the same way the parasite has strategies to modulate the immune response of the insect in its favor, such as the production of nitrite/nitrate, allowing it to establish itself as proposed by Fredensborg et al. (64); Orantes et al. (56); Buarque et al. (65); Soares et al. (18).

The outcome of the microbiota of the insects infected with *T. cruzi* indicates a variation in distribution and abundance between insects that have *T. cruzi* as part of their microbiota and those that do not. This may occur because native bacteria compete for space and resources. Orantes et al. (56) mentions that, in the midgut, an anaerobic environment, bacteria can regulate extracellular glucose levels to facilitate or prevent the colonization of competitors such as *T. cruzi* and by the interaction between insects and microbiota-parasite (18). However, more research needs to carry out in this area.

Knowledge regarding the bacterial intestinal microbiota of triatomines has increased, we know that the associations between symbiont bacteria and the host are varied and necessary for the correct development of the insect (66). Nevertheless, at the moment, we do not know the symbioses between bacteria and the competition that exists between them within the insect, in the same way with the rest of the members of the microbiota such as fungi, archaea, protozoa and viruses as mentioned by Gurung et al. (20). Without leaving aside the immune system of R. ecuadoriensis this responsible for regulating these bacterium communities and protecting the insect from pathogenic microorganisms as mentioned by Buarque et al. (65). On the other hand, the implications for the insect of the microbiota present in the exoskeleton must be considered since these may fulfill some type of symbiotic role for the insect that is still unknown.

The environmental changes that we currently experience can aggravate infectious diseases transmitted by vectors. The increase in temperature allows vectors to expand in colder areas, considering the feeding habits of triatomines, which make them potential vectors of other pathogenic microorganisms. Howell et al. (67); Vieira et al. (53) mention that the bacterium *Serratia marcescens* and the genera *Corynebacterium*, *Clostridium* and *Bartonella* are important human opportunistic bacteria and in this study their presence was found as symbionts of *Rhodnius ecuadoriensis*. Similarly, Orantes et al. (56) reports the presence of the following pathogenic nematodes *Angiostrongylus*, *Heligmosomoides*, *Haemonchus*, *Parastrongyloides* and *Strongyloides*. On the other hand, evidence shows that triatomines are also potential vectors of other types of infectious agents of clinical importance, such as the Hepatitis B *virus*. By feeding on a source of infected blood, insects can transmit the virus up to 6 weeks after accidental inoculation as presumed by Vieira et al. (53); Silverman, (68). However, more research needs to be conducted on these types of interactions since the lack of current information limits their understanding.

According to Pietri et al. (69), other hematophagous insects considered pests are increasingly resistant to chemical control methods. Currently, there is no information on chemical resistance of *R. ecuadoriensis*, but we could experience similar situations in the near future. In addition,, the use of chemical insecticides causes a great environmental impact on human and animal health (48,70),for that reason, research based on bio-controllers is an increasingly present need.

The *Rhodnius ecuadoriensis* microbiota is a fundamental piece of its biology and therefore in the transmission of Chagas disease. It is necessary to knowhow it interacts with its host, the interactions between the intestinal microbiota and *Trypanosoma cruzi*. However, the lack of current information about the different microbial kingdoms that make up the gut microbiota makes it difficult to reach a definitive conclusion. More research is needed to determine how different groups interact with each other and how this affects triatomine.

5.1 Limitations

Given the sample pool methodology, individual information on the insects is lost, in the same way bacteria that present a very low abundance are underestimated, there is still no information on the insects from the other provinces of Ecuador and Peru.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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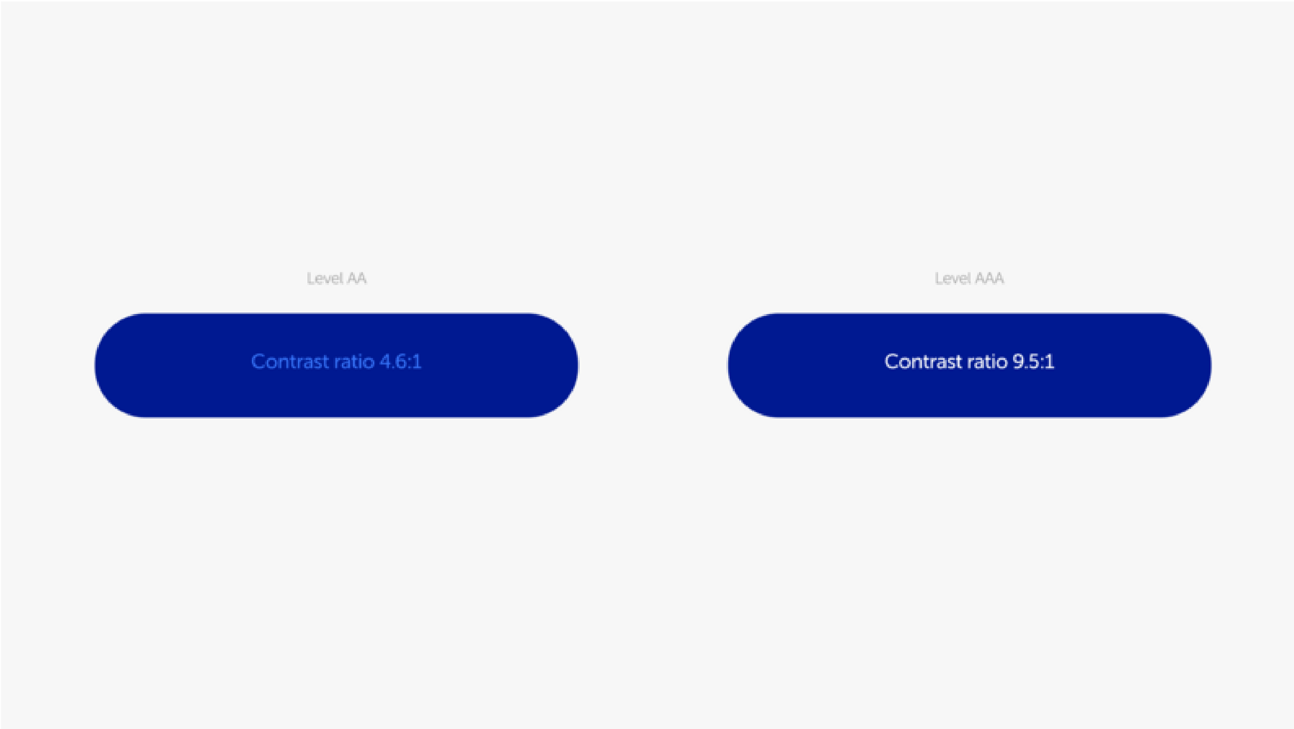

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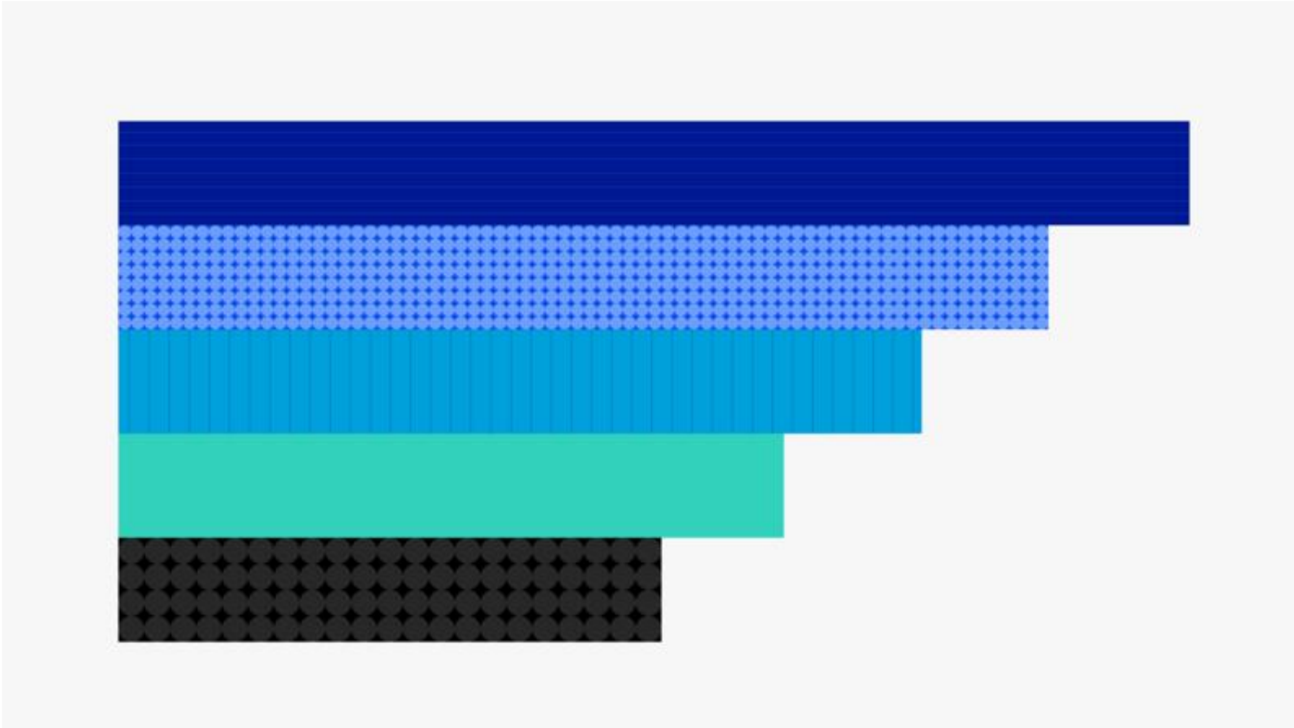

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Data	Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837	(Perdiguero et al., 2015)

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Theses and dissertations	Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago	(Smith, 2008)
Preprint	Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).	(Smith, 2008)
One author	Hesse-Biber, S. (2010). Qualitative Approaches to Mixed Methods Practice. Qualitative Inquiry, 16(6), 455-468. https://doi.org/10.1177/1077800410364611	(Hesse-Biber, 2010)
Two authors	Duvail, S., Hamerlynck, O. (2007) The Rufiji River flood: plague or blessing?. Int. J. Biometeorol. 52, 33–42. https://doi.org/10.1007/s00484-007-0105-8	(Duvail and Hamerlynck, 2007)
More than two authors	Leemhuis C, Thonfeld F, Näschen K, Steinbach S, Muro J, Strauch A, López A, Daconto G, Games I, Diekkrüger B. (2017) Sustainability in the Food-Water-Ecosystem Nexus: The Role of Land Use and Land Cover Change for Water Resources and Ecosystems in the Kilombero Wetland, Tanzania.	(Leemhuis et al., 2017)

Source	Reference list entry	In-text citation
	Sustainability. 9(9):1513. https://doi.org/10.3390/su9091513	
Same author(s); same year	Huang, X-G. (2016a). Electromagnetic fields and anomalous transports in heavy-ion collisions—a pedagogical review. Rep. Prog. Phys. 79 076302. doi: 10.1088/0034-4885/79/7/076302 Huang, X-G. (2016b). Simulating Chiral Magnetic and Separation Effects with Spin-Orbit Coupled Atomic Gases. Scientific Reports. 6:20601. doi: 10.1038/srep20601	(Huang, 2016a, 2016b)
Same author(s); different years	Sedrakian, A. (2007). The physics of dense hadronic matter and compact stars. Progress in Particle and Nuclear Physics. 58(1):168-246. doi: 10.1016/j.ppnp.2006.02.002 Sedrakian, A. (2016). Axion cooling of neutron stars. Phys. Rev. D 93:065044. doi. 10.1103/PhysRevD.93.065044	(Sedrakian, 2007, 2016)
Same first author; different author list	Quimque, M. T., Notarte, K. I., Letada, A., Fernandez, R. A., and Pilapil, D. Y. 4th., Pueblos, K.R., Agbay, J.C., Dahse, H.M., Wenzel-Storjohann, A., Tasdemir, D., Khan, A., Wei, D.Q., Gose Macabeo, A.P. (2021a). Potential Cancer- and Alzheimer's Disease-Targeting Phosphodiesterase Inhibitors fromUvaria alba: Insights from In Vitro and Consensus Virtual Screening. ACS Omega. 6, 8403–8417. doi: 10.1021/acsomega.1c00137	(Quimque et al., 2021a; Quimque et al., 2021b)

Source	Reference list entry	In-text citation
	Quimque, M. T. J., Notarte, K. I. R., Fernandez, R. A. T.,Mendoza,M. A. O., Liman, R. A. D., Lim, J. A. K., et al. (2021b). Virtual screening-driven drug discovery of SARSCoV2 enzyme inhibitors targeting viral attachment, replication, post-translational modification and host immunity evasion infection mechanisms. J Biomol Struct Dyn. 39, 4316–4333. doi: 10.1080/07391102.2020.1776639	
Different authors; same surname	Khan, S. M., Khan, M., Alouffi, A., Almutairi, M. M., Numan, M., Ullah, S., et al. (2023). Phylogenetic position of Haemaphysalis kashmirensis and Haemaphysalis cornupunctata, with Notes on Rickettsia spp. Genes. 14, 360. doi: 10.3390/genes14020360 Khan, Z., Shehla, S., Alouffi, A., Kashif Obaid, M., Zeb Khan, A., Almutairi, M. M., et al. (2022). Molecular survey and genetic characterization of Anaplasma marginale in ticks collected from livestock hosts in Pakistan. Animals. 12, 1708. doi: 10.3390/ani12131708	(Khan Z. et al., 2022; Khan S. M. et al., 2023)
Publishing in a Humanities and Social Sciences journal	Farrell, H. (2012). The Consequences of the Internet for Politics. Annual Review Political Science. 15, 35-52. http://dx.doi.org/10.1146/annurev-polisci-030810-110815	(Farrell, 2012, p. 40)

Source	Reference list entry	In-text citation
Personal communications		“We thank L. Li (personal communication, December, 2018) for noting this ambiguity.”

Vancouver reference style (numbered)

Reference examples for Frontiers’ journals using the Vancouver referencing system can be found below. For more examples of citing other documents and general questions regarding the Vancouver reference style, please refer to Citing Medicine.

- In-text citations in the Vancouver reference style should be numbered consecutively in order of appearance in the text and identified by Arabic numerals in parenthesis.
- Use square brackets for physics and mathematics articles.
- The abbreviation ‘Ref’ should not be used, e.g.: [e.g., (1)] should NOT read [e.g. Ref. (1)].
- Style for comparing a citation should follow the number format, e.g. [cf. (1)]. The same applies when using ‘see’, e.g. [see (46)].
- References should be numbered and listed chronologically, according to the order they appear in the text.

Reference guidelines - Vancouver

Source	Reference list entry	In-text citation
Article in a print journal	1. Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell. (2000) 5:163-172.	(1)

Source	Reference list entry	In-text citation
Article in an online journal	2. Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol. (2013) 4:6. doi: 10.3389/fendo.2013.00006	(2)
Article or chapter in a book	3. Sorenson PW, Caprio JC. "Chemoreception". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.	(3)
Book	4. Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press. (1997). p. 345.	(4)
Abstract	5. Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer. (2002). p. 182–91.	(5)
Website	6. World Health Organization. E. coli. (2018).https://www.who.int/news-room/fact-sheets/detail/e-coli[Accessed March 15, 2018].	(6)

Source	Reference list entry	In-text citation
Patent	7. Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498. (2002).	(7)
Data	8. Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015). http://dx.doi.org/10.5061/dryad.ps837	(8)
Theses and dissertations	9. Smith J. (2008) Post-structuralist discourse relative to phenomenological pursuits in the deconstructivist arena [dissertation/master's thesis]. Chicago (IL): University of Chicago. (2008).	(9)
Preprint	10. Kingma DP, Ba J. Adam: A method for stochastic optimization. arXiv [preprint]. (2014). Available at: https://arxiv.org/abs/1412.6980 (Accessed June 20, 2014).	(10)
Unpublished reference		"We thank L. Li (personal communication,

Source	Reference list entry	In-text citation
		December, 2018) for noting this ambiguity."
Named citation – One author	20. Hesse-Biber S. Qualitative Approaches to Mixed Methods Practice. Qualitative Inquiry. (2010) 16(6): 455-468. doi: 10.1177/1077800410364611	"Hesse-Biber (20) found…"
Named citation – Two authors	43. Duvail S, Hamerlynck O. The Rufiji River flood: plague or blessing?. Int J Biometeorol. (2007) 52:33–42. doi: 10.1007/s00484-007-0105-8	"… as stated by Duvail and Hamerlynck (43)."
Named citation – Multiple authors	44. Leemhuis C, Thonfeld F, Näschen K, Steinbach S, Muro J, Strauch A, et al. Sustainability in the Food-Water-Ecosystem Nexus: The Role of Land Use and Land Cover Change for Water Resources and Ecosystems in the Kilombero Wetland, Tanzania. Sustainability. (2017) 9(9):1513. doi: 10.3390/su9091513	"In research conducted by Leemhuis et al. (44)..."
Multiple citations	8. Yuan S, Yao X, Yang H, Zhang Y, Liu H, Sun J, et al. Research note: genetic diversity of duck circoviruses circulating in partial areas of Guangdong province, southern China. Poult Sci. (2022) 101:102032. doi: 10.1016/j.psj.2022.102032 … 29. Liu H, Li LX,	"… reported in these regions (8, 29, 30), are…"

Source	Reference list entry	In-text citation
	Sun WC, Shi N, Sun XT, Jin NY, et al. Molecular survey of duck circovirus infection in poultry in southern and southwestern China during 2018 and 2019. BMC Vet Res. (2020) 16:80. doi: 10.1186/s12917-020-02301-x 30. Zhang X, Jiang S, Wu J, Zhao Q, Sun Y, Kong Y, et al. An investigation of duck circovirus and co-infection in Cherry Valley ducks in Shandong Province, China. Vet Microbiol. (2009) 133:252–6. doi: 10.1016/j.vetmic.2008.07.005	
Publishing in physics or mathematics	1. Lee YT, Vardi A, Tordjman M. A hybrid self-aligned MIS-MESFET architecture for improved diamond-based transistors. Appl Phys Lett (2020) 117:202101. doi:10.1063/5.0023662	"…including transistors [1]…"

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