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How does anthropogenic impact affect the microbiome in howler monkeys (genus *Alouatta*) in Bolivia ?

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Abstract

The objective of this Master's thesis is to analyze the impact of anthropogenic environments on the gut microbiome of two howler monkey species, *Alouatta sara* et *Alouatta caraya*, in Bolivia. Fecal samples were collected non-invasively from individuals living in natural forest fragments as well as from individuals living in anthropogenic environments, including two rescue centers and one zoo. The extracted DNA was amplified by PCR and sequenced using a metabarcoding approach for the sequencing of the 16S rRNA gene, and a barcoding approach for the sequencing of the cytochrome *b* gene as well as the internal transcribed spacer (ITS2) region. Cytochrome *b* sequences allowed the identification of each fecal samples to either *Alouatta caraya* or *Alouatta sara*. Statistical analyses revealed statistically significant differences in beta diversity in the composition of microbial communities of *Alouatta* howler monkeys between natural and anthropogenic habitats, as well as between species *Alouatta sara* and *Alouatta caraya*. Furthermore, field observations led to the identification of a previously undocumented contact zone between *A. sara* and *A. caraya*, including one individual exhibiting a hybrid phenotype, suggesting a possible interspecific hybridization event. This preliminary work provides the basis for the implementation of a robust and reproducible protocol for species identification and bacterial microbiome analysis in the genus Alouatta, for future research on the subject or for the continuation of this study at the doctoral level.

Résumé

L'objectif de ce mémoire de master est d'analyser l'impact des environnements anthropiques sur le microbiome intestinal de deux espèces de singes hurleurs, *Alouatta sara* et *Alouatta caraya*, en Bolivie. Des échantillons fécaux ont été collectés de manière non invasive auprès d'individus vivant dans des fragments de forêt naturelle ainsi qu'auprès d'individus vivant dans des environnements anthropiques, incluant deux centres de sauvetage et un zoo. L'ADN extrait a été amplifié par PCR et séquencé en utilisant une approche de métabarcoding pour le séquençage du gène 16S rRNA, ainsi qu'une approche de barcoding pour le séquençage du gène du cytochrome *b* et de la région ITS2 (espaceur transcrit interne). Les séquences du cytochrome *b* ont permis d'identifier chaque échantillon fécal comme appartenant soit à *Alouatta caraya*, soit à *Alouatta sara*. Les analyses statistiques ont révélé des différences statistiquement significatives pour la diversité bêta dans la composition des communautés microbiennes des singes hurleurs du genre *Alouatta* entre les habitats naturels et anthropiques, ainsi qu'entre les espèces *Alouatta sara* et *Alouatta caraya*. De plus, des observations sur le terrain ont conduit à l'identification d'une zone de contact auparavant non documentée entre *A. sara* et *A. caraya*, comprenant un individu présentant un phénotype hybride, suggérant un possible événement d'hybridation interspécifique. Ce travail préliminaire constitue la base pour la mise en place d'un protocole robuste et reproductible destiné à l'identification des espèces et à l'analyse du microbiome bactérien chez le genre *Alouatta*, pour de futures recherches sur le sujet ou pour la poursuite de cette étude au niveau doctoral.

Abbreviations

DNA : Deoxyribonucleic acid
RNA : Ribonucleic acid
rRNA : ribosomal RNA
bp : base pair
cytb : Cytochrome *b* gene
ITS : Internal Transcribed Spacer
16S : 16S ribosomal RNA gene
PCR : Polymerase Chain Reaction
EDTA : Ethylene diamine tetraacetic acid
Tris : tris(hydroxymethyl)aminomethane
TBE : Tris-Borate-EDTA
PERMANOVA : Permutational Multivariate Analysis of Variance
PCoA : Principal Coordinates Analysis
NMDS : Non-metric Multidimensional Scaling

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Introduction

For several years, the number of studies on the gut microbiome has been increasing steadily (Sirisinha, 2016). The microbiome is defined as a characteristic microbial community forming a dynamic and interactive micro-ecosystem, integrated within macro-ecosystems including eukaryotic hosts, and possessing its own physico-chemical properties (Berg et al., 2020). Studies have shown that the microbiome plays a key role in the health and survival of the host (Ogunrinola et al., 2020). A microbiome-gut-brain axis influences behavior and brain functions, can provoke neurodegenerative diseases and affect immunity (Loh et al., 2024). These microorganisms form complex communities in a state of dynamic equilibrium and are shaped both by their host's environment and by the host itself (Forum on Microbial Health et al., 2014)

However, numerous factors have been highlighted as potential threats to the stability of these microbial communities (Ogunrinola et al., 2020). The type and quality of the diet significantly influence the gut microbiome, particularly due to fibers that interact directly with intestinal microbes (Makki et al., 2018). Environmental chemicals or antibiotics can also disrupt the ecological balance of the gut microbiome (Chiu et al., 2020; Schwartz et al., 2020). On the other hand, habitat degradation leads to suboptimal diets and stressful conditions that can impact the stability of the intestinal community and affect the health of the species (Amato et al., 2013; Wasimuddin et al., 2022). Habitat degradation can also lead to hybridization between species (Grabenstein and Taylor, 2018) and subsequently cause changes in the microbiome (Wei et al., 2024). Taken to the extreme, captivity is the pinnacle of habitat degradation and of artificialization of the diet, which can lead to the loss of the native gut microbiome (Clayton et al., 2016). In order to investigate the impact of habitat degradation on the microbiome, whether caused by changes in diet or by human-induced hybridization, it is essential to use as model closely related species that co-occur and are subject to anthropogenic pressure. For this reason, I selected as model species the two howler monkeys on *Alouatta sara* and *caraya*, populations of which occur in sympatry in Bolivia (Figure 1) in various environments ranging from undisturbed natural habitats to care centres/zoo.

The genus *Alouatta*

Howler monkeys (genus *Alouatta*) are folivorous-frugivorous primates. They are among the largest platyrrhines and belong to the family Atelidae, alongside *Ateles, Lagothrix* and *Brachyteles* (Cortés-Ortiz et al., 2015b). The IUCN Red List provides the assessment for 14 *Alouatta* species, although the diversity and taxonomic classification of the genus is still controversial (Cortés-Ortiz et al., 2015b). All species of this genus possess an enlarged hyoid bone that forms a kind of resonance chamber, allowing them to produce low-frequency ($<$1 kHz) and long-distance howls that can be heard up to 5 km away (Holzmann et al., 2012; Rumiz, 2014). Howler monkeys occupy a wide range of habitats across South America (Figure 1), from the south of Veracruz State in Mexico to the north of Argentina (Crockett, 1998; Doyle et al., 2021).

Figure 1. Geographical distribution of howler monkeys, genus *Alouatta* (Doyle et al., 2021)

This diversity of habitats exposes them differently to anthropogenic pressures, such as deforestation, forest fragmentation, and agriculture. Although they are classified as ecologically flexible primates due to their broad geographical distribution and the diversity of their habitats, these populations are known for their vulnerability to human disturbances, particularly changes in the quality and availability of food resources as well as hunting, and face the risk of extinction across their entire geographical range (IUCN, 2015, 2020; Estrada, 2015)

These characteristics make them excellent bioindicators of the effects of human activities on ecosystems. Bolivia, rich in ecological diversity and natural resources, harbors significant populations of *Alouatta sara* and *Alouatta caraya*. Studying primate microbiomes is essential for understanding population health, conserving species by assessing their health status in environments undergoing anthropogenic impacts, and comparing microbiome diversity and evolution across multiple species (Forum on Microbial Health et al., 2014).

Alouatta sara

Alouatta sara, or the Bolivian Red Howler Monkey, belongs to the group of red howlers (comprising *A. seniculus, A. arctoidea, A. macconnelli, A. juara, A. puruensis, and A. sara*), characterized by a reddish brown fur and endemic to Bolivia and southern Peru (Botting, gust; IUCN, 2020; Rumiz, 2014). It was once considered a subspecies of *Alouatta seniculus* but is now regarded as a distinct species (Figure 2)(Minezawa et al., 7 16; Stanyon et al., 1995).

The IUCN describes the geographical range of *A. sara* as extending from the department of Pando in the South, along the Andes mountain range, and returning towards central Bolivia further East, including the entire basin of the Río Beni and extending to the Mamoré-Guaporé region (IUCN, 2020).

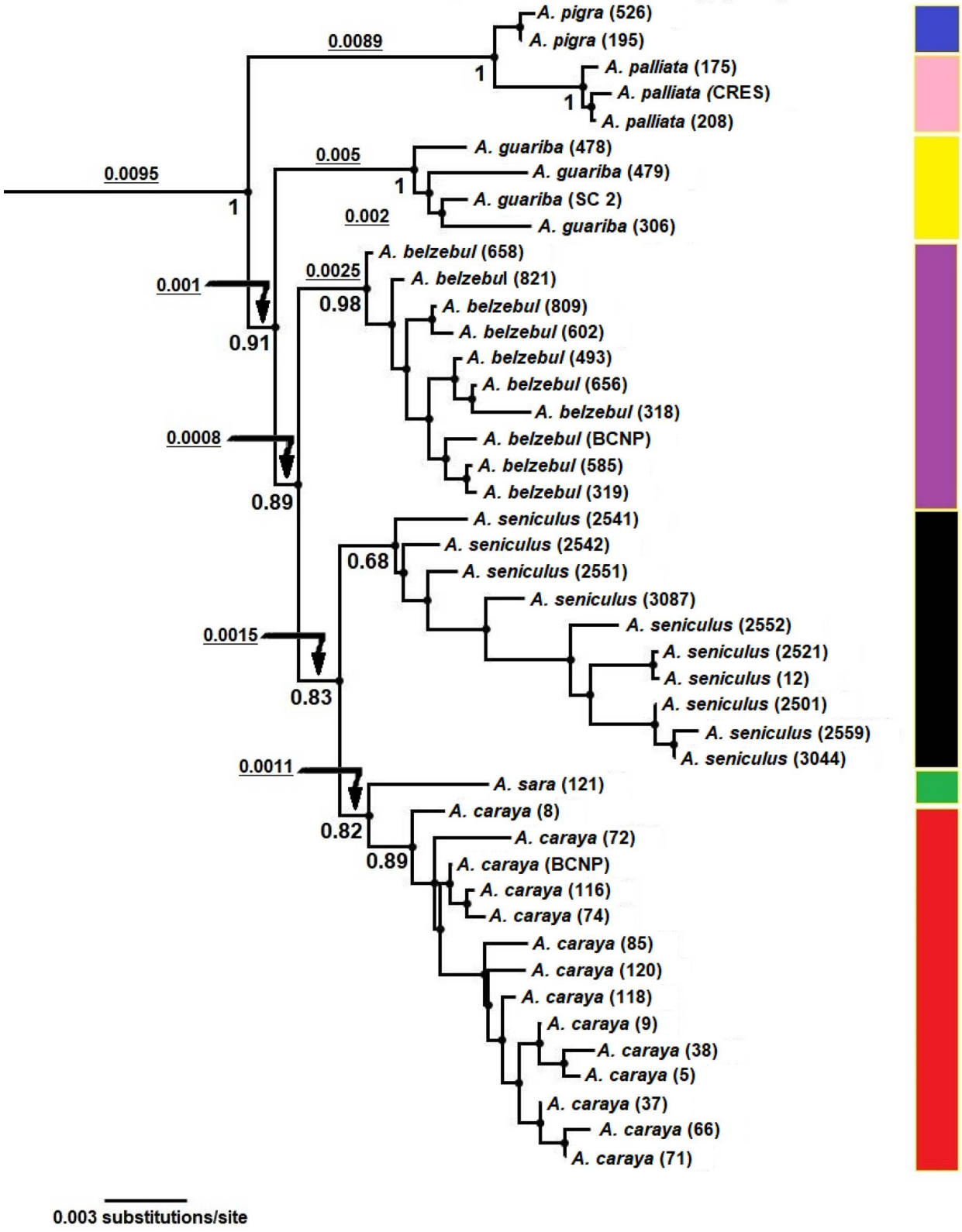

Figure 2. Bayesian phylogenetic tree of the genus *Alouatta*, modified from Doyle et al. (2021). Node support values are Bayesian posterior probabilities, whereas branch lengths are displayed underlined (the outgroup clade was cropped out). Colored rectangles on the right highlight species boundaries.

Alouatta caraya

Alouatta caraya, or the Black-and-gold Howler Monkey, differs from other howler monkey species by the presence of distinct sexual dichromatism (Cortés-Ortiz et al., 2015a). Females are straw-colored to reddish brown, with darker olive back and the male have a shiny black coat (Rumiz, 2014). Its range is quite broad, covering the central-western part of Brazil, eastern Bolivia and Paraguay, northeastern Argentina, and finally, the northwestern part of Uruguay (IUCN, 2015).

Potential hybridization

Hybrid zones between *Alouatta* species have been studied in Brazil and Mexico. Molecular evidence of hybridisation in *Alouatta* was first presented for the northernmost Central American howlers *A. palliata* and *A. pigra* (Cortés-Ortiz et al., 2007). At the other latitudinal extreme of *Alouatta* distribution, populations of *A. caraya* and *A. guariba clamitans* were found in sympatry in the Argentinean Province of Misiones (Di Bitetti et al., 1994; Agostini et al., 2008, 2010) and the Brazilian states of Paraná (Gregorin, 2006; Aguiar et al., 2007, 2008) and Rio Grande do Sul (Bicca-Marques et al., 2008; Dias et al., 2015). Although contact zones between other *Alouatta* species were reported (Cortés-Ortiz et al., 2015a), no further efforts have been made in these areas to investigate whether hybridisation occurs and the impacts of such gene flow on their ecology, social organisation, and populations.

One such example is the contact zone between *Alouatta sara* and *Alouatta caraya* in the Río Yacuma, Beni Department in northern Bolivia (Büntge and Pyritz, 2007). Büntge and Pyritz (2007) emphasised the difference in the pattern of pelage coloration typical of each species in groups foraging in the same area. However, they did not report the existence of mixed groups - with individuals of both species forming a single social unit; or groups composed by individuals of a mixed pattern of pelage colouration. Therefore, the ecological aspects of these sympatric groups were never studied and the potential hybridization between these species was never investigated.

Materials and Methods

1. Field work

1.1. Premise and development of the project

The development of this project is based on close collaboration with the Noel Kempff Mercado Museum of Natural History, located in Santa Cruz de la Sierra, Bolivia. A friend introduced me to Damián I. Rumiz, a biologist specializing in biodiversity, particularly of primates, who became my main collaborator at the museum. Together, with the support and guidance of my supervisor Jean-François Flot and my advisor Felipe E. Silva, we defined the project's objectives and established the methodology to follow. Thanks to Damián, we were able to set up five sampling sites in natural environments as well as three in anthropogenic areas (Table 1). This approach allowed us to study the microbiome of both species not only in their natural habitat but also under high anthropogenic pressure.

1.2. Study area

Five forest fragments located on both banks of the Río Grande were initially planned for study. However, site No. 4 was inaccessible due to heavy rainfall that flooded the trail leading to the sampling area. The five natural sampling sites are located near the city of Santa Cruz de la Sierra, in the Santa Cruz Department, Bolivia (Figure 3) (Table 1). This department is situated in the eastern part of the country (13°40'-20°20'S; 57°30'-64°40'W) and is significantly impacted by expanding agricultural activities. This factor contributed to the loss of 2,31 million hectares of humid primary forest betseen 2002 and 2022, representing 47% of the total tree cover loss during this period (source: Global Forest Watch). The region has a heterogeneous terrain, ranging from low-elevation areas and a few mountains in the central and eastern sectors- wich do not exceed 1250m- to the Andean region, where elevations reach nearly 3100m (Maillard et al., 2020). Additionally, samples were collected in two wildlife rescue centers as well as at the zoo of Santa Cruz.

Map code	Field Site	Size (ha)	Closest village/city (distance)	River bank (Río Grande)	Taxon
1	Jose Miterer	500	Buena Vista (3km)	West	*A. sara*
2	Juan Deriba-Anke	700	Santa Rosa del Sara (1.9km)	West	*A. sara*
3	Jardin Botánico	217	Santa Cruz de la Sierra (5km)	West	*A. caraya*
4	Ibbecken	1000	Santa Cruz de la Sierra (48km)	West	Mixed groups
5	San Miguelito	4683	San Antonio de Lomerío (40km)	East	Mixed groups
6	Zoo	/	Santa Cruz de la Sierra (0km)	West	*A. caraya & sara*
7	AFASI	/	Warnes (19km)	West	*A. sara*
8	CAD	/	Santa Cruz de la Sierra (0km)	West	*A. caraya*

Table 1. Survey sites for *A. sara*, *A. caraya*, and potential mixed groups.

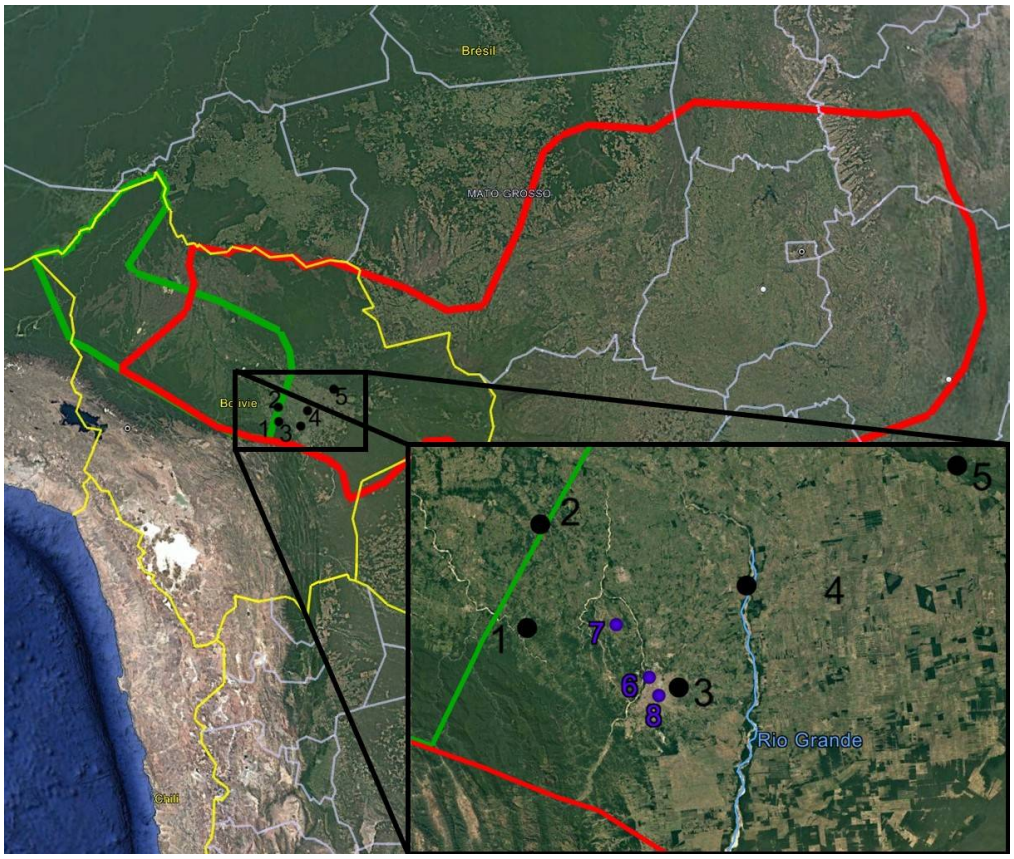

Figure 3. Natural (black dot) and anthropogenic (purple dot) sampling sites - Delimitation of the distribution of A. sara (in green) and A. caraya (in red)

1.2.1. Natural sites

The four natural sites visited are all private properties owned by individuals committed to their conservation. Each site experiences varying degrees of external pressure. Sites No. 1, 2, and 3 belong to the tropical/subtropical moist forest biome, whereas Site No. 5 falls within the tropical/subtropical semi-humid/dry forest biome (Pacheco et al., 2016)

Sampling site No. 1 (17°29'S 63°39'W) is located in the municipality of Buenavista, Ichilo Province, Santa Cruz Department, Bolivia. The study site covers an area of approximately 30 ha within a 500 ha forest parcel owned and preserved by its proprietor (Figure 4). Active efforts are made to prevent hunting of the species within the reserve; however, it remains a persistent threat and represents one of the main anthropogenic pressures (Estrada, 2015; Büntge and Pyritz, 2007) on *A. sara*, the only howler monkey species inhabiting this region.

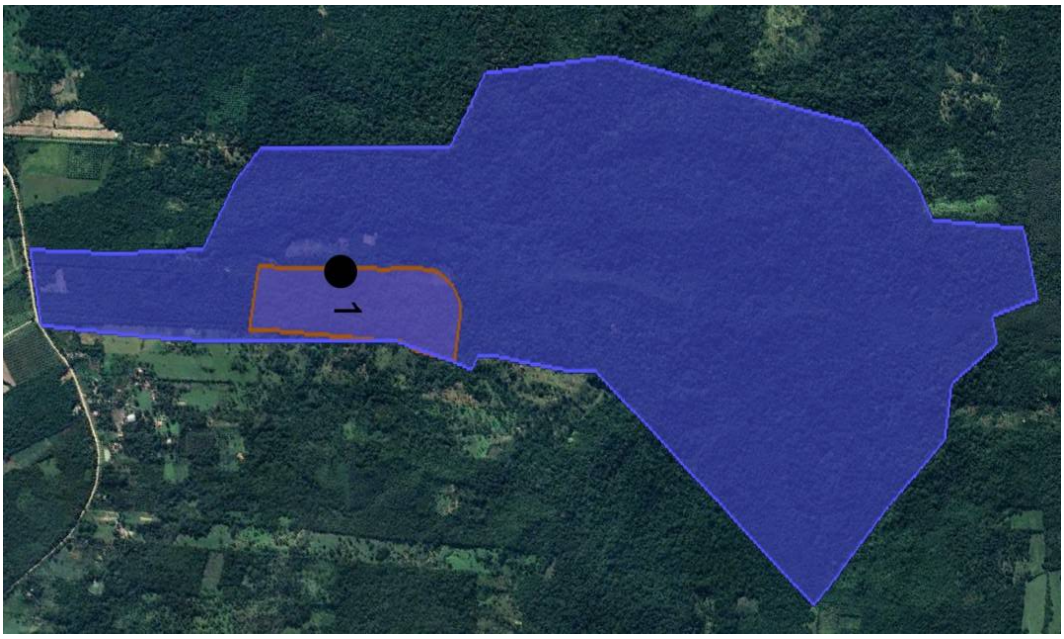

Figure 4. Field site n°1 delimitation - Jose Miterer.
The blue area represents the total forest plot owned by the owner. The sampling area was focused in the orange zone. The black dot represents the field site number

Sampling site No. 2 (17°6'S 63°34'W) is located in the municipality of Santa Rosa del Sara, Sara Province, Santa Cruz Department, Bolivia. The study site covers an area of approximately 750 ha (Figure 5). The landowners, Dieter and Anke, combine agricultural activities, particularly cattle ranching, with environmental conservation. Juan Deriba's property preserves one of the few remaining parcels of continuous natural vegetation (forests and savannas) in the northern region (Pinto-Ledezma et al., 2014). Once again, efforts are made to combat hunting, although some individuals still manage to enter the property. As with site No. 1, only *A. sara* is present in the region.

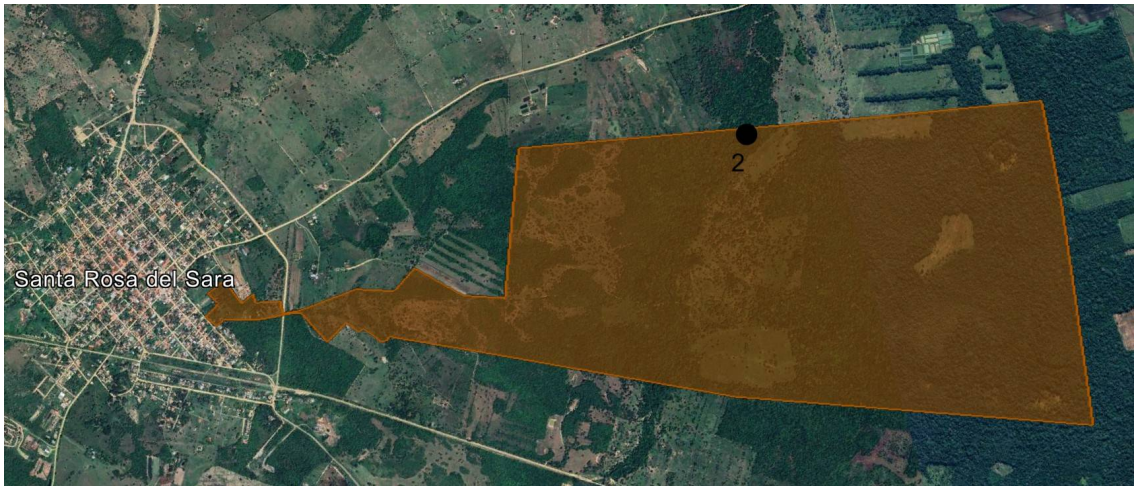

Figure 5. Field site n°2 delimitation - JuanDeriba-Anke
The orange area represents the sampling area. The black dot represents the field site number

Sampling site No. 3 (17°45'S 63°4'W) is located in the municipality of Santa Cruz de la Sierra, Andrés Ibáñez Province, Santa Cruz Department, Bolivia. The study site covers an area of 217 ha (Figure 6). The Municipal Botanical Garden, founded in 1984, is an important site for biodiversity. It houses over 500 plant species as well as a diverse fauna . The garden allows animals to live in an environment similar to their natural habitat. However, they live in semi-captivity, as the entire

garden is enclosed by walls to protect the site from human activities beyond its boundaries. This site allowed the sampling of specimens from *Alouatta caraya*

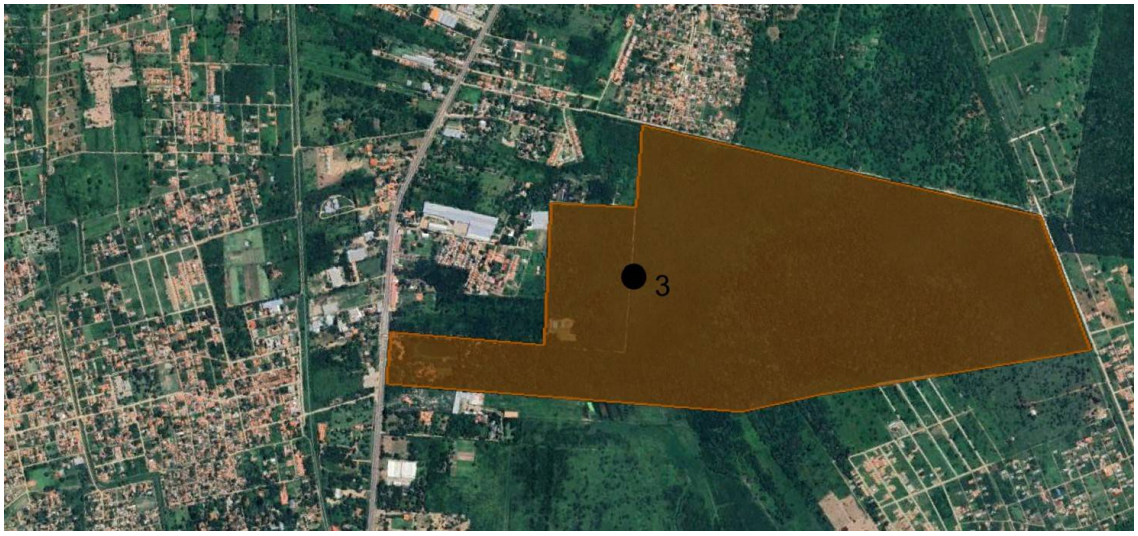

Figure 6. Field site n°3 delimitation - Jardin Botanico.
The orange area represents the sampling area. The black dot represents the field site number

Sampling site No. 4 (17°24'S, 62°45'W) was not accessible due to heavy rainfall in the area, which made the site unreachable and therefore excluded it from this study.

Sampling site No. 5 (17°1'S 61°51'W) is located in the municipality of San Antonio de Lomerío, Ñuflo de Chávez Province, Santa Cruz Department, Bolivia. The study site covers an area of approximately 4800 ha, and unlike neighboring properties often deforested for agriculture, San Miguelito Jaguar Conservation Ranch has preserved more than 90% of its natural forest, providing a refuge for a rich and diverse fauna (Figure 7) (Ochoa, 2017).

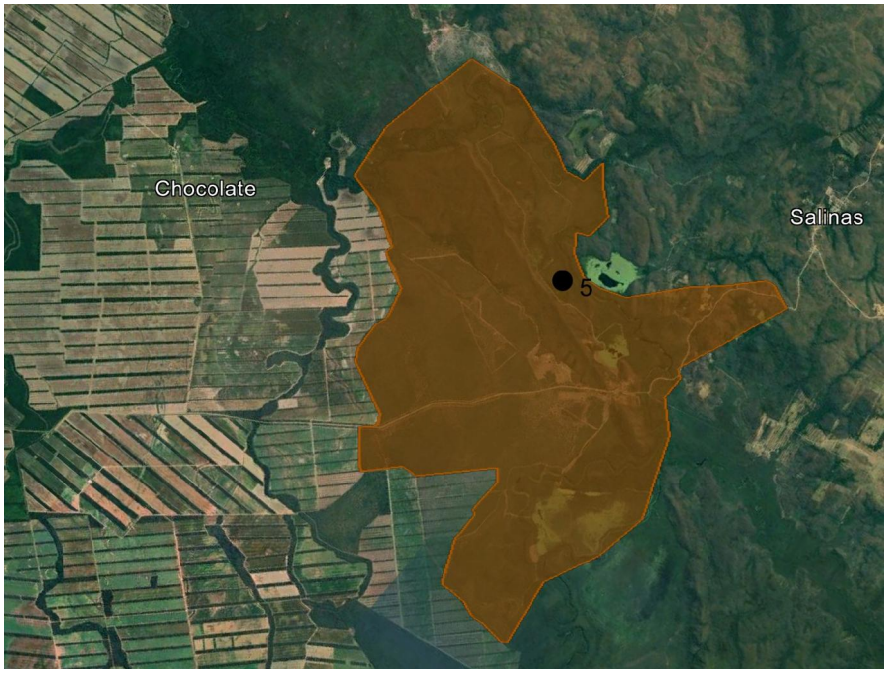

Figure 7. Field site n°5 delimitation - San Miguelito
The orange area represents the sampling area. The black dot represents the field site number

This ranch is an interesting example of conservation integrated with human activities, notably

cattle ranching. It manages to combine livestock farming while maintaining one of the highest jaguar densities in Bolivia. Thanks to the conservation efforts in place, this site boasts a lush fauna and flora. However, it remains vulnerable to external threats such as deforestation, agricultural expansion, and hunting. Beyond the numerous animal and plant species residing within this site, we find the two species of interest in this study, *A. sara* and *A. caraya*.

1.2.2. Anthropogenic sites

In Bolivia, several institutions are dedicated to wildlife protection and the rehabilitation of wild animals. To compare the microbiomes of captive and wild species and analyze the impact of living conditions on the microbiome, it was crucial to find sampling sites for individuals in captivity. Thanks to Damián I. Rumiz, I was able to connect with the Santa Cruz Municipal Zoo and two wildlife rescue centers, allowing me to collect samples from these locations.

Sampling site n°6 (17°45'S 63°11'W) is located at the Santa Cruz de la Sierra Municipal Zoo, in the Andrés Ibáñez Province, Santa Cruz Department, Bolivia. This zoo houses the two studied howler monkey species, *Alouatta sara* and *Alouatta caraya*, allowing the collection of samples from a highly anthropogenic environment (Table 2).

Species	Sample ID	Sex	Age	Weight (kg)	Dietary supplements	Number of individuals in cage
A. caraya	19	Female	Juvenile	3.2	Yes	Alone - quarantine
A. sara	20	Female	Adult	3.5	Yes	Alone - quarantine
A. sara	21	Male	Adult	9.2	Yes	In group (2 individuals) - quarantine
A. sara	22	Female	Juvenile	3.8	Yes	Alone (quarantine)
A. caraya	23	Male	Adult	11.9	No	In group with individual 31 - exposed
A. sara	24	Female	Adult	3.6	No	Alone - exposed
A. caraya	31	Female	Adult	/	No	In group with individual 23 - exposed

Table 2. Description of the living conditions of primates in captivity at the zoo.

The primate diet at the zoo is based on food derived from conventional human agriculture. According to the list provided by the zoo, their diet includes the following foods: banana, cucumber, melon, squash, apple, grape, watermelon, bean, pea, carrot, heart of palm, corn, broccoli, whole grain bread, boiled egg, nuts, almonds, forage, alfalfa, spinach, parsley, celery, and seasonal fruits.

Additionally, a combination of three dietary supplements and pre/probiotics was administered to certain primates in poor health during my visit for sample collection. Prebiotics are defined by the International Scientific Association for Probiotics and Prebiotics (Gibson et al., 2017) as "a substrate that is selectively utilized by host microorganisms, conferring a health benefit," and probiotic bacteria are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." (Cremon et al., 2018). These supplements include:

- B-complex vitamins, containing all eight B vitamins;
- Aminomix, a solution consisting of amino acids, glucose, and electrolytes (salts);
- Organew, a probiotic and prebiotic containing live yeast, FOS (fructo-oligosaccharides), MOS (mannan-oligosaccharides), as well as vitamins and amino acids (`https://vetnil.com.br/produto/organew-r`);

Sampling site n°7 (17°30'S 63°17'W) is the AFASI Foundation (Amigos de la Fauna Silvestre), located in the municipality of Santa Cruz de la Sierra, Andrés Ibáñez Province, Santa Cruz Department, Bolivia. This Bolivian organization is dedicated to wildlife protection. The foundation provides refuge to animals rescued from illegal trafficking and inappropriate captivity conditions. This refuge allowed for the collection of samples from *Alouatta sara* in captivity (Table 3).

Species	Sample Number	Sex	Age	Weight (kg)	Dietary Supplements
A. sara	26	Female	8 months	1.235	Yes
A. sara	27	Male	2 years	3.556	Yes
A. sara	28	Male	8 months	1.040	Yes
A. sara	29	Male	8 months	0.919	Yes

Table 3. Description of captive primates' living conditions at the AFASI foundation.

The primates' diet at the foundation is also based on food derived from conventional human agriculture. The list of food provided includes: lettuce, spinach, broccoli, carrot, cucumber, sweet potato, zucchini, cauliflower, beetroot, cake, papaya, apple, peach, and pineapple.

Dietary supplements and nutritional additives are provided to the primates. Among these, Organew, a probiotic and prebiotic, is included, along with lactose-free milk and Cerelac, a cereal-based infant formula.

Sampling site n°8 (17°47'S 63°9'W) is the Centro de Atención y Derivación de Fauna Silvestre en Bolivia (CAD), located in the municipality of Santa Cruz de la Sierra, Andrés Ibáñez Province, Santa Cruz Department, Bolivia. CAD is an animal rescue center dedicated to the rehabilitation of wild animals rescued from illegal trafficking and mistreatment. This refuge allowed for the collection of samples from *Alouatta caraya* living in captivity (Table 4).

Species	Sample Number	Sex	Age	Weight (kg)	Dietary Supplements
A. caraya	42	/	8 months	/	No
A. caraya	43	Male	infant	/	No

Table 4. Description of captive primates' living conditions at the CAD center.

The diet at the center is similar to that of the zoo and AFASI. During my visit to the center, I was able to observe a typical meal provided to the primates present at the center (Figure 8).

Figure 8. Meal prepared for primates, containing cucumber, watermelon, banana, carrot, celery, pineapple, boiled egg, heart of palm, parsley, and quinoa soup

1.3. Primate tracking

Field research was conducted between February 15 and March 31, 2024. In each of the four natural sites studied, the primate tracking method was identical. Since these sites are private properties, the first step was to gather information from the landowners. They provided valuable insights, such as areas frequently visited by howler monkeys (e.g., near certain fruit trees they favor), territory boundaries, access points, and other relevant details.

The necessary equipment for primate tracking included:

- A satellite GPS (e.g., GPSMAP® 64) to track routes, retrace paths, and record key locations.
- A machete, to facilitate movement through dense forest.
- A pair of binoculars, allowing detailed observation of the primates to accurately describe their fur coloration, sex, age, etc.

The first few days on each site were dedicated to observation and familiarization with the area. During this period, a landowner or a local worker accompanied us to help identify potential traces of the primates and to navigate the terrain. Once this phase was completed, I was able to continue tracking the primates independently. Since howler monkeys typically defecate simultaneously shortly after waking up, the initial plan was to venture deep into the forest, locate a group of howler monkeys, follow them until nightfall, set up camp near them, and collect fecal samples the next morning after they awoke. However, logistical challenges necessitated an adaptation of this approach. Transporting all the necessary equipment (food, stove, hammock, etc.) through dense tropical forest proved difficult. A new method was therefore implemented, dividing the day into two phases and allowing most of the equipment to be left at the accommodations provided by the landowners.

Phase One: Early Morning Tracking The first phase involved waking up at 4:00 AM and reaching the heart of the forest by 4:45-5:00 AM, before the first light of day. At dawn, howler monkeys often produce their characteristic calls to mark their territory. These calls, audible over several kilometers, provided a means to locate the group and navigate toward them before they stopped vocalizing. Once the group was located, we waited at the base of the trees where the primates were perched. Howler monkey behavior follows a simple yet cyclical pattern: at dawn, they begin with territorial calls, then quiet down, observe their surroundings, move between nearby trees, and start foraging. Afterward, a latency phase occurs before they defecate simultaneously. At this moment, sample collection became possible.

Phase Two: Afternoon Tracking The second phase was initiated when howler monkeys did not vocalize in the morning, or if searches in the forest after sunrise yielded no results. In such cases, tracking resumed in the afternoon, focusing on scanning the canopy in hopes of locating a group. If a group was found, we followed them deep into the forest until dusk. After sunset, when the last light faded, howler monkeys generally ceased movement and settled in a familiar area, often rich in food, where they spent the night. Once I had followed the group until nightfall and confirmed they would not move further, I recorded their position on my satellite GPS and placed reflective cords on the trees where they were perched. After this setup, I returned to my accommodation. The following morning, I ensured I returned to the exact GPS location by 4:30 AM to be present at sunrise and resume tracking if the primates moved again. The reflective ropes installed the previous night facilitated locating the area. It was crucial to avoid using bright flashlights to illuminate the canopy, as this could prematurely awaken the primates, disrupting their perception of dawn and causing them to move, making tracking significantly more difficult—particularly given the complete darkness on the forest floor. While the ground remained pitch dark, the primates, positioned in the treetops, had an easier time moving.

Through this method, the different natural sites were systematically explored, and all samples were collected in a non-invasive manner.

1.4.Sample collection

For sample collection, we followed the "Primate Microbiome Project Protocols" (`https://www.primatemicrobiome.org/protocols`) as well as the "Environmental Dropping Sampling Kit" (SYL006) from the Sylphium company. Fecal samples were collected immediately after defecation (or as soon as possible) using the sterile built-in plastic spoon included in each collection tube. The sampling tubes (Figure 9) contained a preservation solution that allows the sample to be stored for several months without the need for alcohol or freezing. This system provides a major advantage in tropical environments by reducing the need for additional equipment.

Each tube was opened only once during sample collection and remained sealed until laboratory analysis. Approximately 1 gram of material was collected to ensure an adequate amount for DNA extraction, which requires between 0.2 and 0.3 grams of fecal material. The feces were split in half in order to collect from all parts of the excrement, and a portion was collected and placed inside the tube (Figures 10 & 11). Whenever possible, in addition to sample collection, information on group size was recorded, as well as the age and sex of individuals. For each group of observed primate, the location of the GPS and the composition of the group (number of adult males, adult females and immature individuals) were documented, along with individual characteristics (e.g., coat color). Each sample was linked to an identification sheet insofar as the individual was observed before collection (Annex 1 -S1) and the sample name was marked directly on a label affixed to the tube in the form MM20240228.1.01, meaning: initials-dates-sampling location-sample number. Due to the fact that I was alone for the collection and, in general, the entire group defecates at the same time, it was not always possible to precisely identify each individual. Each tube was carefully placed in an individual plastic bag, sealed with Parafilm, and labeled—both the tube and the plastic bag carry matching identification labels.

Figure 9. Sampling tube

Figure 10. Feces from *A. caraya* at the Jardin Botanico site

Figure 11. *A. caraya* feces split open at the botanical garden

1.5. Permits, authorizations, and collaboration

Upon arriving in Bolivia, I went to the Noel Kempff Mercado Museum of Natural History to meet, Kathia, and Luzmilla and sign a letter of intent to formalize the collaboration between the Noel Kempff Mercado Museum of Natural Science and ULB (Annex 1 - S2). This collaboration enabled me to obtain various authorizations, granting me access to private and restricted areas

that would otherwise be inaccessible without official permission, such as the AFASI and CAD rescue centers, the Zoo, and the Botanical Garden.

The NKM Museum was responsible for submitting an official request (Annex 1 - S6) before receiving authorization to access these sites. Gaining access to the Botanical Garden proved more challenging than expected. However, with the support of the Faculty of Agricultural Sciences at the Gabriel René Moreno Autonomous University, and after signing a liability waiver, access was eventually granted.

Finally, the NKM Museum also provided me with the necessary authorization to leave the country by issuing the zoosanitary certificate granted by SENASAG (annex 1 S7).

2. Laboratory work

2.1. DNA extraction

DNA extraction from the collected fecal samples was performed using the Environmental DNA Isolation Kit (SYL002) from Sylphium (reference SYL002/100/000, lot 240130), specifically designed for the isolation of environmental DNA. The process began with the preparation of tubes containing the phase separation solution (S2) and the precipitation solutions (S3 and S4), which are necessary for processing the samples and removing contaminants. The tubes were then cooled to -20°C to precipitate the DNA, leaving salts and other unwanted substances in solution. After this step, the tubes were centrifuged at 13,000 g to remove the supernatant, followed by an additional washing step. The extracted DNA was then eluted in the preservation solution provided by the Sylphium® kit and stored at -20°C.

To assess the quantity and quality of the extracted DNA, two tests were performed: a fluorometric quantification test using the Qubit™ 4 Fluorometer (Invitrogen by Thermo Fisher Scientific®) and a purity assessment test using the Nanodrop Onec (Thermo Fisher Scientific®). The results showed that the extracted DNA contained impurities. The 260/280 ratio, which should ideally be close to 1.8, was lower, suggesting the probable presence of proteins or other contaminants absorbing at 280 nm, such as phenols or organic compounds. Additionally, the 260/230 ratio, which should ideally range between 2 and 2.2, was also lower, indicating the presence of organic solvents, salts, or residual chemicals from the extraction process.

When working with fecal samples, it is important to note that the samples themselves may contain contaminants such as phenols, fatty acids, salts, or proteins, which can influence the results if these substances are not entirely removed during DNA extraction.

The extraction protocol was adjusted to optimize the process. Since the samples had been collected several months before laboratory processing, cell lysis had already occurred. However, we attempted to incubate the sample tubes to release more DNA from cellular material and thus increase the final DNA yield. However, no significant difference was observed in the DNA quantity after extraction. The amount of dissolved sample was also modified, first reduced from 50 µl to 5 µl, then increased from 50 µl to 250 µl. While the level of contaminants remained unchanged, increasing the dissolved sample volume resulted in a higher final DNA yield. Consequently, all samples were extracted using 250 µl, instead of the initially planned 50 µl. Finally, rather than performing a single final wash, the wash solution volume was reduced from 900 µl to 500 µl, and this step was repeated twice to maximize the removal of contaminants.

2.2. PCR amplification

Once extracted, DNA was amplified through polymerase chain reaction (PCR). The advantage of working with fecal samples is that it allows the recovery of DNA from both the bacterial populations present in the feces and the host primate, via shed epithelial cells. All PCRs performed for this work were carried out using the two-step PCR method (Figure 12), developed within the Evolutionary Biology and Ecology laboratory at ULB. This method consists of a first PCR step in which the target sequence is amplified using the selected primers. In the second step, a barcode is attached to the PCR products generated in step one (Figure 13). Each barcode is linked to a specific sample, which allows for the sequencing of all samples simultaneously while still knowing which sequence comes from which sample, and therefore which bacterium originates from which individual in the context of microbiome analysis.

For each PCR, 1 µL of forward and reverse barcode was deposited in the cap of the reaction tube prior to the preparation of the mixture. This allowed the barcodes to dry while the reaction mix was being prepared. The following components were then added to the tube:

- 1 µL of extracted DNA (approximately 10ng)
- 4,5 µL of nuclease-free water

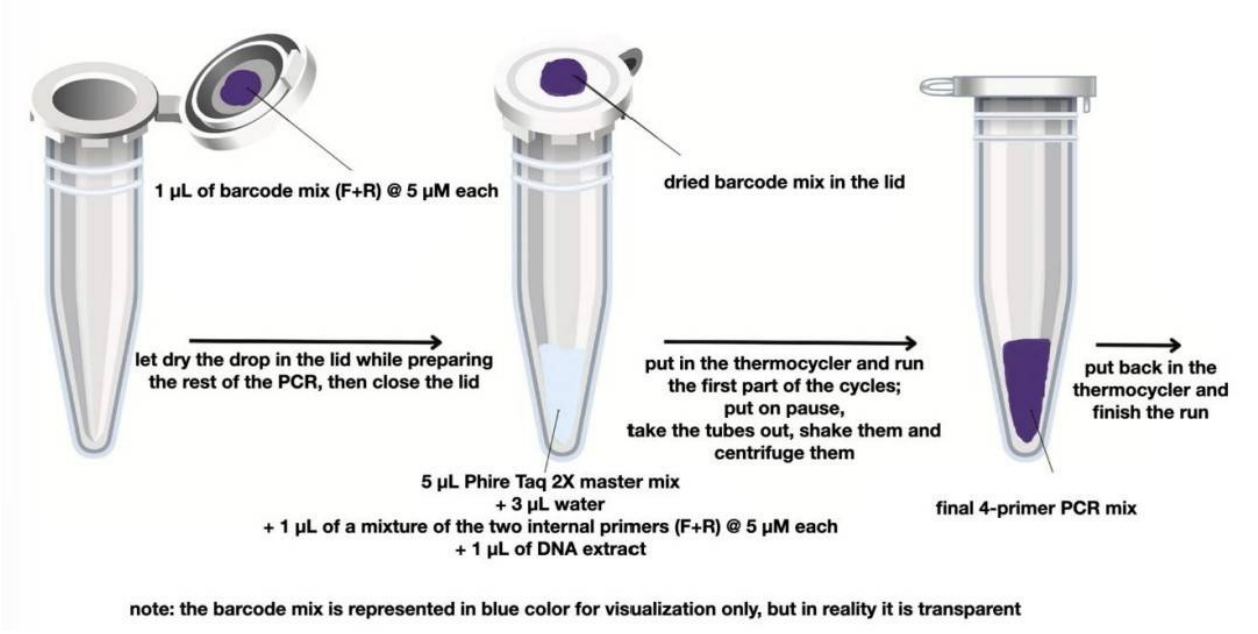

Figure 12. Schematic representation of the two-step PCR protocol used

- 6 µL of Phire Taq polymerase mix MM 2×
- 1 µL of a mixture of forward and reverse primers, each at 5 µM, as recommended by the Phire Taq protocol

The tubes were then placed in the thermocycler, following a cycling program specifically optimized for the gene region targeted by each primer pair. During the hold phase, the tubes were removed and briefly inverted to allow the liquid to reach the cap, where the barcodes have dried. The tubes were then centrifuged to bring the mixture back down, and returned to the thermocycler for the second PCR step, during which the barcodes are incorporated into the amplified product.

Typically, a linker called a "tail" (in purple) was added to the primer (in dark), along with a barcode (in blue) that allows identification of the sample from which each DNA sequence originates. As a result, between 68 and 72 base pairs must be added to each side of the amplified amplicon. For instance:

GATAC-AGAACGACTTCCATACTCGTGTGA-TTTCTGTTGGTGCTGATATTGC-GGTAACAAATCATAAAGATATTGG

It is important to note that the low A260/280 and A260/230 ratios indicated a certain level of impurity in the DNA extracts, which made the PCRs particularly challenging and inconsistent. The reactions no longer responded to the standard conditions of the protocol. Some PCRs were successful with only a small amount of DNA, while others required significantly higher quantities. For some samples, no amplification could be achieved despite numerous attempts to optimize the reaction conditions.

Step 1 :

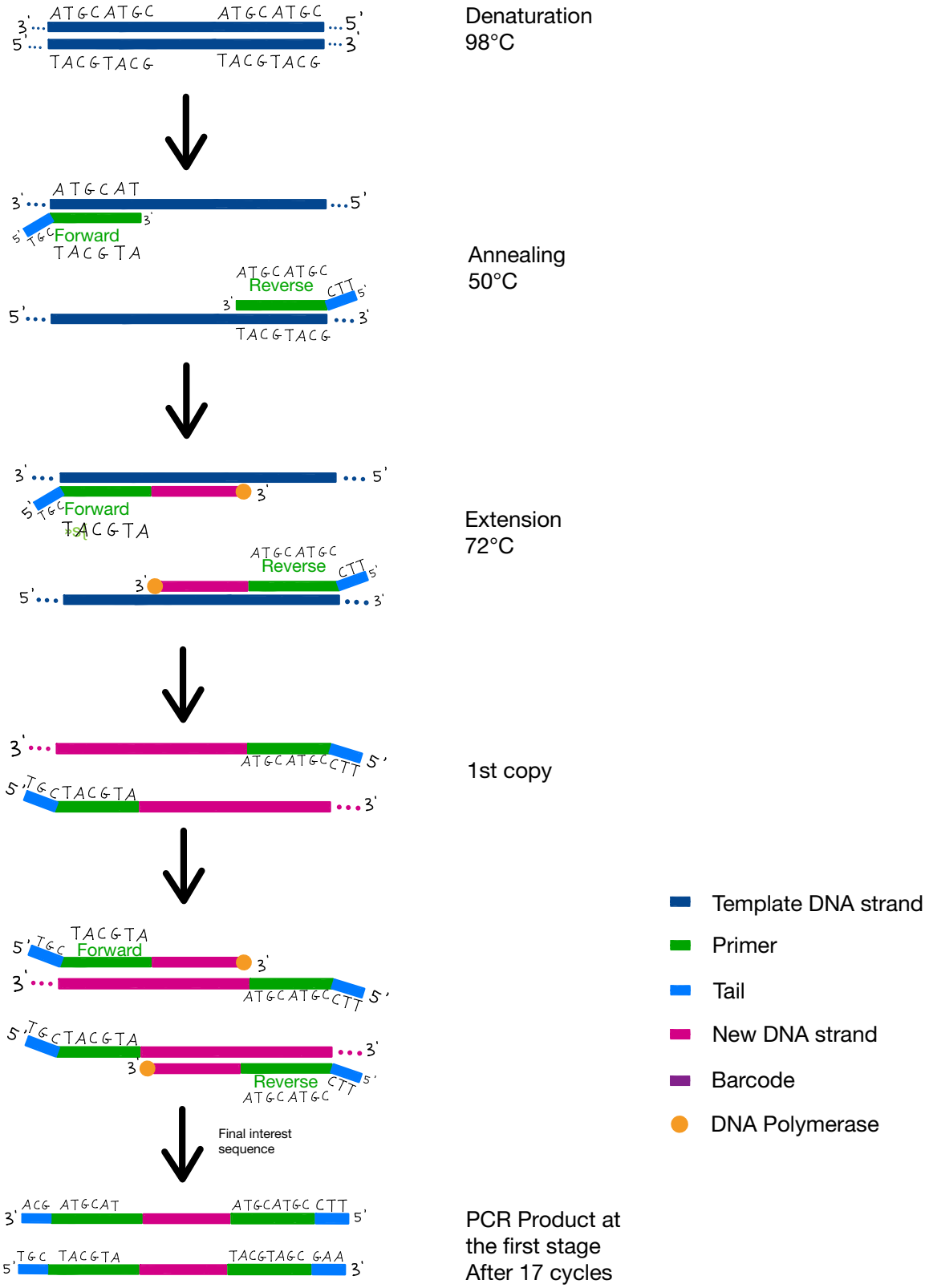

Figure 13. Diagram illustrating the two-step PCR amplification process (1st step)

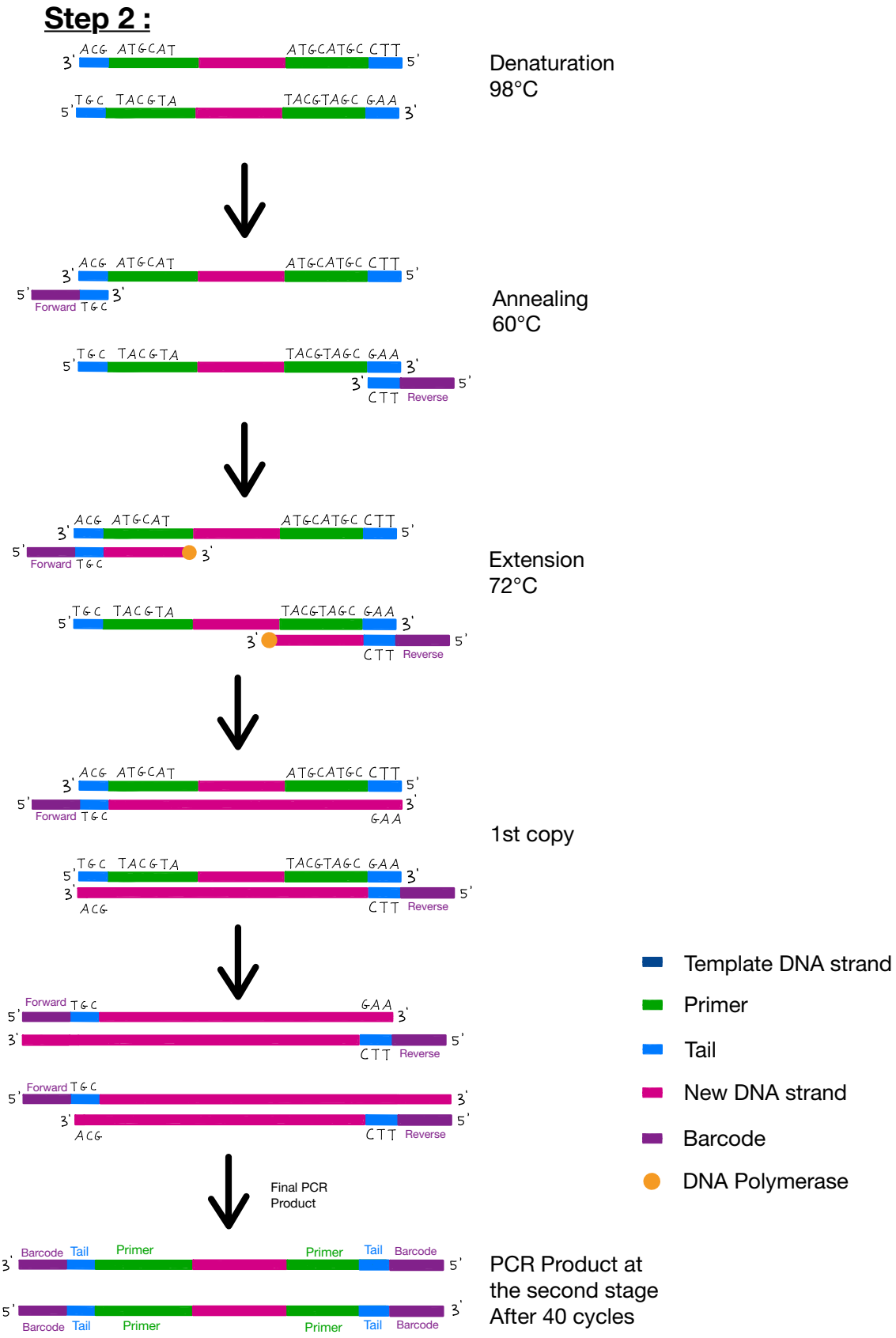

Figure 14. Diagram illustrating the two-step PCR amplification process (2nd step)

2.2.1. PCR Amplification of the 16S rRNA Gene

Microbiome analysis was carried out using the 16S metabarcoding technique which allows for a detailed characterisation of microbial communities. Using the 16S ribosomal RNA gene enables the study of bacterial phylogeny and taxonomy (16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory, n.d.). This gene is sufficiently long to allow for meaningful analysis and is present in most bacteria, retaining the same function throughout evolution ((Lamoril et al., 2008)). The primers used to amplify the 16S rRNA gene fragment were as follows ((Eden, 1991):

27f-L (Forward): 5'- TTTCTGTTGGTGCTGATATTGC-AGAGTTTGATCCTGGCTCAG-3'
1492r-L (Reverse): 5'-ACTTGCCTGTCGCTCTATCTTC-GGTTACCTTGTTACGACTT-3'

The blue sequences correspond to the tails added to the primers, which are required for the two-step PCR method.

Table 5. PCR program used for amplification of the 16S DNA fragment.

Cycles	Step	Temperature	Duration
	Initial denaturation	98°C	300 sec
17×	Denaturation	98°C	8 sec
	Annealing	50°C	8 sec
	Extension	72°C	40 sec
	Hold	10°C	∞
23x	Denaturation	98°C	8 sec
	Annealing	60°C	8 sec
	Extension	72°C	40 sec
	Hold	10°C	∞

The expected amplicon size for the 16S marker is approximately 1600bp.

2.2.2. PCR amplification of the ITS2 region

The Internal Transcribed Spacer 2 (ITS2) region of nuclear ribosomal DNA was the first molecular marker used in this study for the identification of the animal species investigated. ITS2 contains both conserved regions enabling the use of universal primers and a high level of sequence variability at the species level, making it a reliable phylogenetic marker (Yao et al., 2010; Wiemers et al., 2009). The primers used for the PCR amplification of the ITS2 sequences in *Alouatta sara* and *Alouatta caraya* were universal primers commonly employed for this region in animals (Yao et al., 2010; White et al., 1990) to which we added, highlighted in blue, the tail sequences required for the implementation of the two-step PCR method:

ITS3-L (forward) : 5' - TTTCTGTTGGTGCTGATATTGC-GCATCGATGAAGAACGCAGC-3'
ITS4-L (reverse) : 5'-ACTTGCCTGTCGCTCTATCTTC-TCCTCCGCTTATTGATATGC-3'

Table 6. PCR program used for amplification of the ITS2 region.

Cycles	Step	Temperature	Duration
	Initial denaturation	98°C	300 sec
20×	Denaturation	98°C	8 sec
	Annealing	50°C	8 sec
	Extension	72°C	40 sec
	Hold	10°C	∞
20x	Denaturation	98°C	8 sec
	Annealing	60°C	8 sec
	Extension	72°C	40 sec
	Hold	10°C	∞

The expected amplicon size for the ITS2 region is approximately 500–550bp.

2.2.3. PCR amplification of a cytochrome *b* gene fragment

The cytochrome *b* gene (*cytb*), located in the mitochondrial DNA, represents the second marker used in this study for the molecular identification of the animal species under investigation. This gene is commonly used both for species identification and in phylogenetic studies (Hsieh et al., 2001a,b; Irwin et al., 1991; Kuwayama and Ozawa, 2000; Su et al., 1999) One of the main challenges when working with DNA extracted from fecal samples lies in the fact that this DNA is often highly degraded. In this context, we chose to target a partial sequence of the cytochrome b gene. Primer selection was initially based on primers previously validated by the scientific community (Parson et al., 2000), and specifically used to amplify a portion of the cytochrome b gene in *Alouatta caraya* (Oklander et al., 2021). The primers used are as follows:

L14816 (forward): 5'-CCATCCAACATCTCAGCATGATGAAA-3'
H15173 (reverse): 5'-CCCCTCAGAATGATATTTGTCCTCA-3'

However, none of the PCRs were successful during the initial tests (Figure S8)

Using the Primer3 program, we observed that primers L14816 and H15173 showed several mismatches with the known cytochrome b gene sequences of *Alouatta sara* and *Alouatta caraya*. In theory, such mismatches could still allow for successful amplification. However, due to the low quality and purity of the extracted DNA, these primers proved to be ineffective. To confirm whether this inefficacy was indeed due to the primers' incompatibility with the conditions imposed by the DNA quality, a new round of PCR was performed using a temperature gradient on three samples that had consistently yielded positive results with the ITS primers (Figure S9 ; Table S1).

To solve this problem, we directly designed our own primers. To do so, the known sequences of the cytochrome b gene from *Alouatta sara*, *Alouatta caraya*, and *Alouatta seniculus*, the species presumed to be the closest relative of the first two (Figure 2), were downloaded from the NCBI database. The full cytochrome b sequence obtained was 1339 base pairs (bp) long. All sequences were then aligned using the Primer3 program, and we searched for a region with no, or very few, mismatches between the sequences of *Alouatta sara* and *Alouatta caraya* (Figure 15 ; Figure 16) in order to design our primers.

The forward primer starts at base pair 510 and is located in a fully conserved region among the three species used (*A. sara*, *A. caraya*, and *A. seniculus*). The reverse primer starts at base pair 864 and lies in a nearly conserved region. A mismatch is present between *A. sara* and *A. caraya*, but it is not expected to hinder amplification. We are therefore targeting a 355 bp fragment of the cytochrome b gene, which should allow for successful PCR amplification even from degraded DNA containing inhibitors, as is often the case in fecal samples. The addition of tails (in blue) brings the total fragment length to 399 bp.

-Cytb-L (forward): 5' - TTTCTGTTGGTGCTGATATTGC-CAGCCATCCCATACATCGGA-3'
-Cytb-L (reverse) : 5'-ACTTGCCTGTCGCTCTATCTTC-TTAATGTGGGGTGGGGTGTT-3'

Subsequently, the PCRs (Figure S10), but with a consistently low success rate, requiring constant adjustments of the DNA quantities or dilutions of the DNA extracts used.

2.3. Electrophoresison an agarose gel

To verify that the PCR amplification of the target DNA fragments was successful, agarose gel electrophoresis was performed. The agarose gels were prepared using the following protocol: 1g of agarose (reference: 35-1020 & 443666A, VWR®) was mixed with 100mL of 1X TBE buffer (Tris, Borate, EDTA) in an Erlenmeyer flask and heated for approximately two minutes until the agarose was completely dissolved. After removal from the microwave and slight cooling, 7µL of

Figure 15. Forward primer selected using Primer3

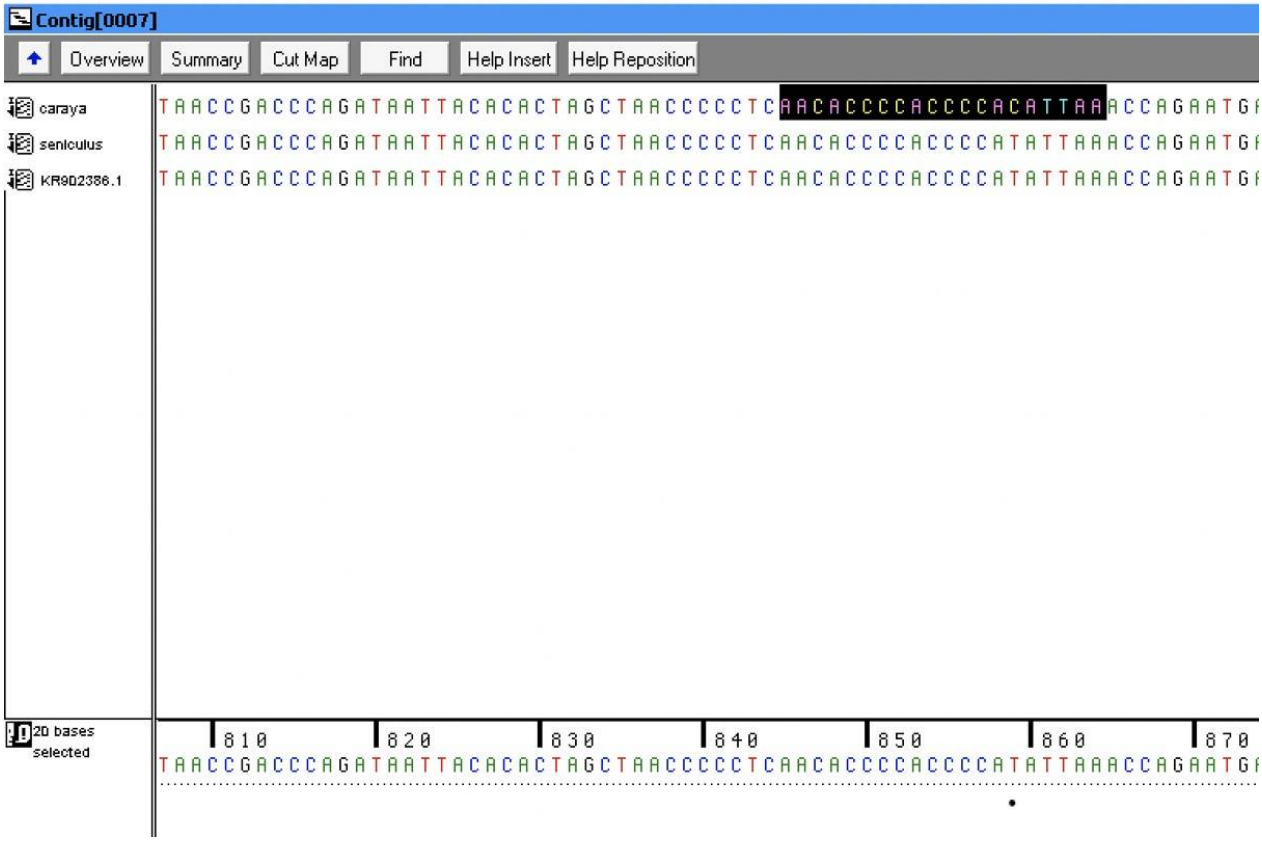

Figure 16. Reverse primer selected using Primer3

Midori Green Stain (reference: MG-04, NIPPON Genetics EUROPE GmbH) was added and mixed to ensure homogenization.

The gel was then poured into the electrophoresis tray, fitted with one or two combs depending

on the number of wells needed, and left at room temperature until fully solidified. The tray was subsequently placed into the electrophoresis chamber and covered with 1X TBE buffer. The combs were then removed, and the PCR products, the DNA ladder (O'GeneRuler Express DNA Ladder, Thermo Scientific®), and the negative control were loaded into the agarose gel wells, each in a volume of 3µL.

Electrophoresis was carried out at a voltage of 125V and a current of approximately 250mA (reference: PS500X, Hoefer Scientific Instruments®), allowing for the separation of DNA fragments according to size. At the end of the run, the power was turned off, the tray was removed from the chamber, and the gel was carefully placed onto a UV transilluminator (Gel Doc 2000, BIO-RAD®) to visualize the bands corresponding to the amplified DNA fragments

2.4. Clean up of amplified DNA

In order to prevent nanopore saturation of the flowcells during sequencing, it is essential to remove unwanted DNA fragments from the pool. To achieve this, a clean-up step is performed after the construction of the pool. Each band obtained by agarose gel electrophoresis is compared to a reference DNA ladder to estimate the relative concentration of the amplified products. Based on this estimation, the samples are grouped into three concentration categories (low, medium, or high), which determines the volume to be used for building an equimolar pool: respectively 1.5 µL, 1 µL, or 0.5 µL per sample.

The next step is to determine the fragment sizes to be removed. For sequencing of the 16S ribosomal RNA gene, the target fragment size is approximately 1500 bp. For fragments amplified from the ITS2 region or the cytochrome b gene, the expected sizes range between 400 and 550 bp. The clean-up is carried out using the SPRI Select Beads method (Beckman Coulter), which selectively removes unwanted DNA fragments based on their size.

For 16S amplicons, the total pool volume is multiplied by 0.5 (Figure 17) to calculate the appropriate volume of beads to add, allowing retention of fragments equal to or larger than 1500 bp while removing shorter ones. For ITS2 and cytochrome b, a ratio of 0.8 is used, as a 0.7 ratio resulted in excessive loss of fragments of interest (Figure 17).

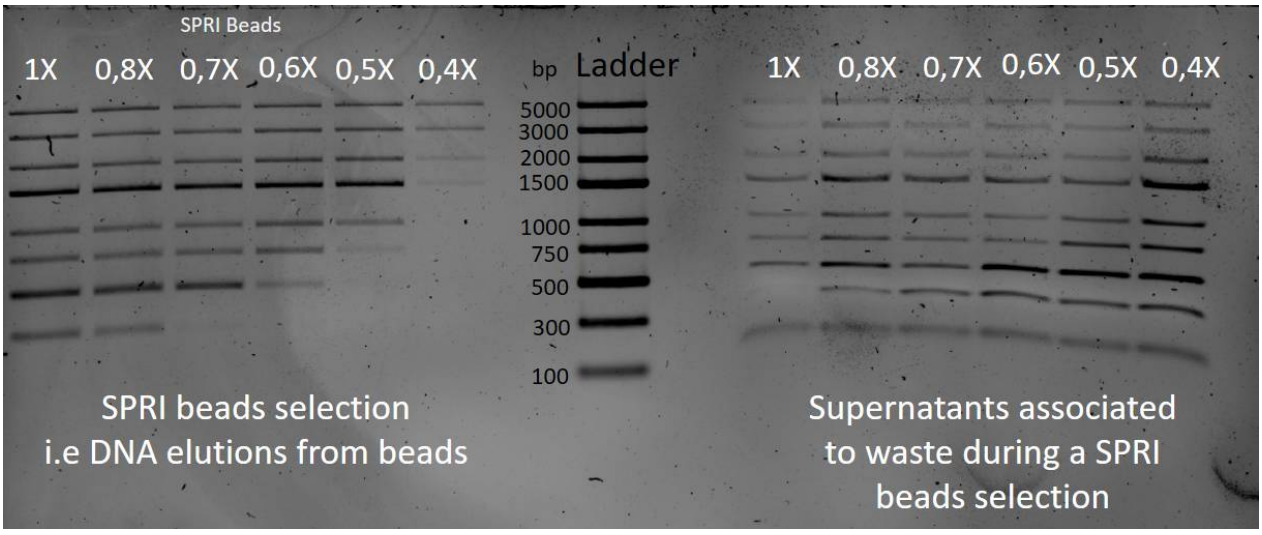

Figure 17. Experiment illustrating SPRI bead-based size selection. Experiment and picture by Florence Rodriguez, Evolution Biologique et Ecologie/ULB lab technician

This clean-up step is critical, as it removes large amounts of short DNA fragments, which are often highly abundant (cf band intensity at the bottom of the gel, Figure S10) and could either compromise sequencing or significantly reduce its quality.

The clean-up protocol is relatively straightforward: the predetermined volume of beads is added to the solution containing the DNA fragments of interest. The tube is then placed on a magnetic

stand, allowing the beads, bound to the DNA, to attach to the magnet. The supernatant is discarded, and 180 µL of 85% ethanol is added. The mixture is incubated at room temperature for 30 seconds to remove contaminants. After a second removal of the supernatant, elution is performed by removing the tube from the magnet and adding at least 20 µL of molecular biology grade water. The eluate is mixed by pipetting to resuspend the beads and incubated for one minute. Finally, the tube is returned to the magnet, and the purified DNA is recovered and transferred to a new storage tube.

Once the clean-up is complete, fluorometric quantification is carried out using the Qubit™ 4 Fluorometer (Invitrogen by Thermo Fisher Scientific®) to determine the concentration of the pool in ng/µL. Finally, the volume of the cleaned-up pool required to reach the recommended 200 fmol for sequencing library preparation is calculated using the Bioline online tool.

2.5. DNA sequencing

Sequencing was performed using the Nanopore technology developed by Oxford Nanopore Technologies®. This method is characterized by its ability to identify, in real time, each nucleotide base of the DNA fragments being analyzed. This continuous reading is made possible by nanopores embedded in an electro-resistant membrane, through which an electric current flows. As a DNA fragment, composed of a sequence of nucleotides A, T, G, and C, passes through the nanopore, each base causes a specific disturbance in the current. These variations are then interpreted by basecalling algorithms, which reconstruct the nucleotide sequence (How Nanopore Sequencing Works, n.d.) (Deamer et al., 2016)

All sequencing in this study was carried out using the ligation sequencing kit V14 (ref. SQK-LSK114). As previously mentioned, 200 fmol were taken from the pooled sample containing all DNA extracts.

Prior to adapter ligation, the DNA fragment ends must be specifically prepared. This step was carried out using the NEBNext Ultra II End Repair/dA-Tailing Module (NEB, E7546), which performs three essential modifications of the DNA ends:

- Blunt-end repair : cohesive ends (overhangs) are converted into blunt ends, compatible with ligation.
- A-tailing: an adenosine is added to the 3' end of each strand. This step enables directional ligation, as the Nanopore adapters have a complementary thymine at the 5' end.
- Phosphorylation 5' : 5' phosphorylation: a phosphate group is added to the 5' end of the DNA, which is essential for the ligase to form a covalent bond with the adapter.

The sequencing adapters were then ligated to the prepared DNA ends using the NEBNext Quick Ligation Module (NEB, E6056). This step enables the DNA fragments to be recognized and captured by the nanopores during sequencing.

A purification step was subsequently performed using AMPure XP magnetic beads, in order to remove excess unligated adapters, residual enzymes, and short DNA fragments. The purified DNA was then eluted in an elution buffer, forming the final library ready to be loaded onto the flow cell.

Finally, the library was loaded onto a PromethION flow cell, and sequencing was initiated using the MinKNOW software, which controls the device, monitors the sequencing run, collects raw data, and performs basecalling to convert the electrical signal into nucleotide sequences (Deamer et al., 2016; Wick et al., 2018)

2.5.1. Metabarcoding and barcoding

In this project, two complementary molecular sequencing approaches were implemented, depending on the analytical objectives. A metabarcoding strategy was applied for the sequencing of the 16S ribosomal RNA gene (16S rRNA), in order to assess the bacterial diversity present in

environmental samples. This method relies on high-throughput sequencing of a universal marker gene, amplified from DNA extracted from collected faecal samples, allowing the simultaneous identification of multiple taxa (Santos et al., 2020)

In parallel, a barcoding approach, a powerful taxonomic tool, was used to target the Internal Transcribed Spacer (ITS) region and the cytochrome b (cytB) gene, with the aim of identifying the primate species under study. Unlike metabarcoding, this strategy is designed to identify one organism at a time, based on DNA derived from a single individual or well-characterized sample (Antil et al., 2023) (Wick et al., 2018)

2.5.2 Data processing

Sequencing was performed using a PromethION device in combination with the MinKNOW 2.2 software developed by Oxford Nanopore Technologies. This system records ionic current variations caused by the nucleotides of the DNA as they pass through nanopores (Deamer et al., 2016). These variations generate a raw signal known as a squiggle, which forms the basis of the sequencing data(Deamer et al., 2016; Ip et al., 2015).

This raw signal corresponding to the ionic current is recorded in a FAST5 file and is then interpreted during the basecalling step, performed using Guppy, a software tool also developed by Oxford Nanopore Technologies for the bioinformatic post-processing of sequencing data (Wick et al., 2018; Vierstraete and Braeckman, 2022).The output of this step is a FASTQ file containing DNA strand reads, each associated with a quality score (Kahlke, 2025; Vierstraete and Braeckman, 2022; Wick et al., 2018).

Since all samples are loaded simultaneously onto a flowcell, a demultiplexing step is required (Wick et al., 2018; Vierstraete and Braeckman, 2022). This process is also handled by Guppy and is based on the detection of barcodes added to each DNA fragment. It allows the alignment of all obtained sequences and their classification according to barcode, thereby assigning each read to the correct sample(Vierstraete and Braeckman, 2022).

For 16S sequencing, the software Emu was used to analyze the reads, identify, and quantify the microbial species present in each sample (Curry et al., 2022). In the case of ITS and cytB sequencing, the Amplicon Sorter software was employed. This tool compares sequences based on their similarity and length, allowing the construction of consensus sequences(Vierstraete and Braeckman, 2022). These consensus sequences were then compared to known DNA sequences using the BLAST tool, in order to associate each DNA sequence and thus each sample with a specific species.

3. Statistical analyses

I relied on two complementary measures: alpha diversity and beta diversity.

3.1. Alpha diversity

Alpha diversity assesses the richness and distribution of microbial species within a single sample. It provides insights into the internal structure of the microbiome and enables comparisons between groups based on their diversity and/or species abundance (Xia and Sun, 2017; Xia et al., 2018). To assess the effect of habitat type (natural vs anthropogenic) or the effect of the species (*A.sara*vs *A. caraya*) on microbial diversity, two alpha diversity indices, Shannon and Simpson, were calculated for each sample. The null hypothesis (H0) states that there is no significant difference in microbial diversity between the two habitat types/the two specie, while the alternative hypothesis (H1) proposes that microbial diversity varies according to habitat/specie.

3.1.1. Indices used

- Shannon index (H'): combines both species richness (number of species) and evenness (distribution of abundances). A higher Shannon index reflects greater diversity, indicating a large

number of species that are relatively evenly distributed. the formula used is (Spellerberg and Fedor, 2003) :

$$H = -\sum_{i=1}^{n} p_i \ln p_i$$

Where p_i is relative abundance of species i in the community.

- Simpson index (D): When one species strongly dominates, the index approaches 1. Conversely, when abundances are evenly distributed among species, the index tends toward 0. Unlike the Shannon index, the Simpson index gives more weight to the most abundant species. the formula used is (Berger and Parker, 1970) :

$$D = 1 - \sum_{i=1}^{S} p_i^2$$

Where p_i is relative abundance of species i in the community.

3.1.2. Associated statistical tests

Before comparing diversity indices between groups, data normality is assessed using the Shapiro-Wilk test. If the data are normally distributed (p-value $>$ 0,05), a Welch's t-test is (Welch, 1947). This is a variant of the classic Student's t-test that does not assume equality of variances (homoscedasticity) between groups. It maintains good statistical power and reduces the risk of errors (Ruxton, 2006).In our analyses, this version was systematically used whenever the assumption of normality was met. If the data do not follow a normal distribution (p-value $<$ 0,05), the Wilcoxon-Mann-Whitney test is used instead, as it does not rely on distributional assumptions. The null hypothesis (H0) tested in both cases is that alpha diversity (Shannon or Simpson) does not differ significantly between the groups compared.

3.2. Beta diversity

Beta diversity compares the bacterial composition between different samples. It is based on the calculation of a distance matrix that quantifies the dissimilarity between pairs of samples (Xia and Sun, 2017; Xia et al., 2018).

3.2.1. Distances used

In microbiome analysis, data are both compositional and sparse. In this context, Euclidean distance is not appropriate, and alternative distance metrics are preferred, notably the Bray-Curtis distance, which accounts for relative abundances (quantitative), or the Jaccard index, which considers only species presence or absence (qualitative)(Xia et al., 2018). These two distances can produce similar community structures. In our case, a Mantel test revealed a strong correlation between Bray-Curtis and Jaccard distances (r = 0.9883), confirming that both metrics yielded a highly similar organization of the data. For our study, the Bray–Curtis dissimilarity index will be used throughout.

3.2.2. Visualization methods

From the distance matrix, two main approaches allow for the visualization of potential differences in microbial composition between groups :

- PCoA (Principal Coordinates Analysis) : projects the distance matrix into a reduced-dimensional space. Axis 1 captures the greatest proportion of variance, followed by axis 2, and so on.
- NMDS (Non-Metric Multidimensional Scaling) : provides an ordinal representation based solely on the rank order of distances, without preserving their absolute values.

3.2.3. Associated statistical test

PERMANOVA (Permutational Multivariate Analysis of Variance) is a multivariate statistical test applied to the distance matrix. It is used to evaluate the effect of different factors, such as species, environment, or their interaction, on microbial composition. The null hypothesis (H0) tested is that microbial composition does not differ significantly between the groups defined by the tested factors. PERMANOVA determines whether the observed differences in community composition are statistically significant.

Results

1. Influence of habitat on the microbiome

1.1. Alpha diversity

The Simpson index (Figure 18) measures bacterial species abudance, giving greater weight to the most abundant taxa. Visually, the median value appears slightly higher for individuals living in anthropogenic habitats, but intra-group variability is greater among samples from the natural environment

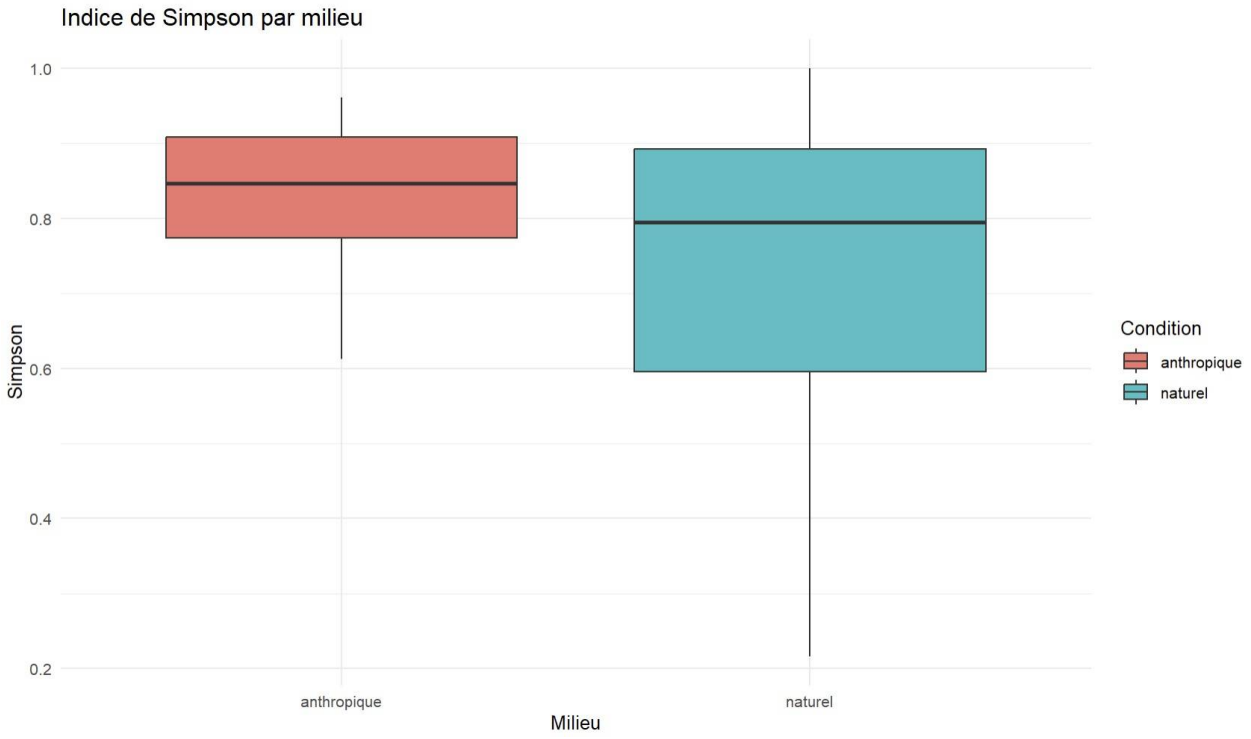

Figure 18. Simpson index by habitat type

The Shapiro-Wilk test revealed that the distribution was not normal for samples from the natural habitat ($p = 0.0088$), requiring the use of a non-parametric test. The Wilcoxon–Mann–Whitney test yielded a p-value of 0.258, indicating that no significant difference in bacterial dominance was detected between the two habitat types.

The Shannon index (Figure 19), which accounts for both richness and evenness by giving weight even to rare species, was also analyzed.

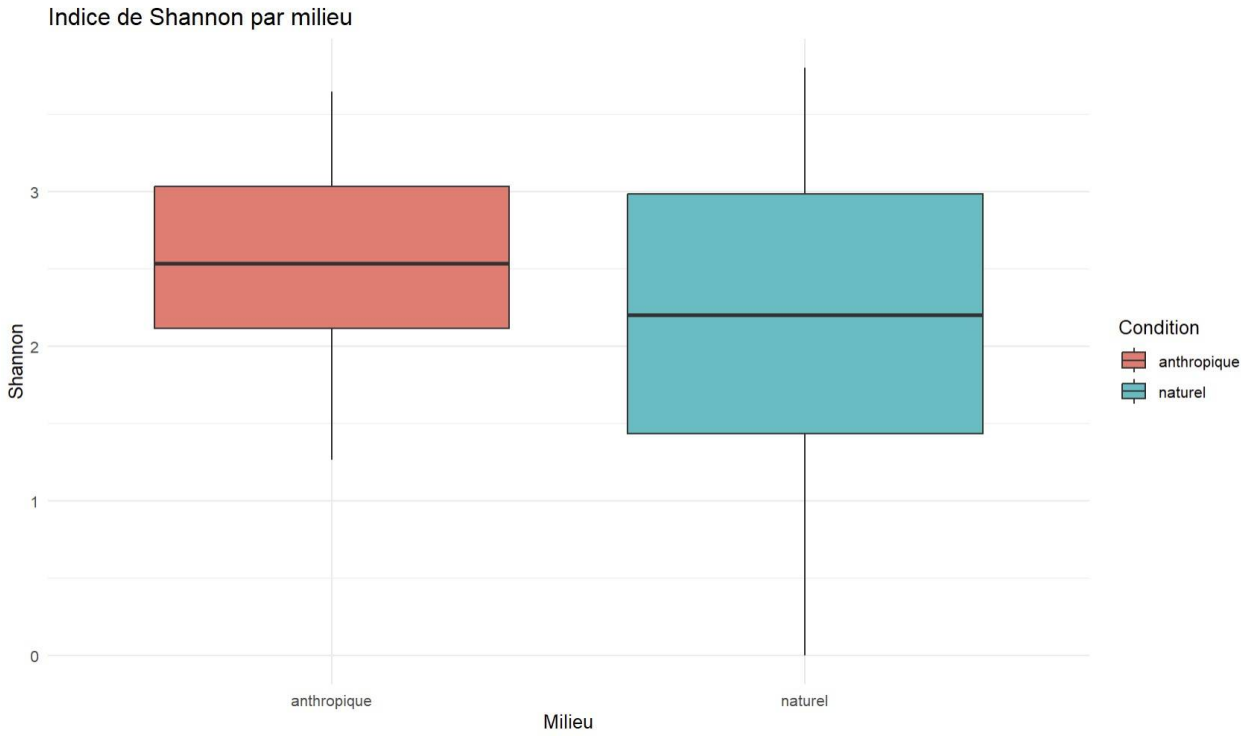

Figure 19. Shannon index by habitat type

In this case, both distributions followed a normal distribution (Shapiro-Wilk p $>$ 0.05), allowing the use of a Welch's t-test (which is appropriate for unequal sample sizes) (Xia et al., 2018; Ruxton, 2006). The mean was slightly higher in the anthropogenic habitat (2.55) compared to the natural habitat (2.18), but the resulting p-value (0.2265) indicated that this difference was not statistically significant. The 95% confidence interval [-0.25 ; 0.99] included zero, suggesting that the observed difference could be attributed to random variation.

1.2. Beta diversity

The NMDS (Figure 20) shows a stress of 0.187, indicating a good-quality ordination (stress $<$ 0.2) (Xia et al., 2018). Visually, individuals from natural habitats (blue points) form a relatively compact cluster, while those from anthropogenic habitats (red points) appear more dispersed, suggesting greater variability in microbial composition within the latter group. To test the significance of this pattern, a PERMANOVA was performed. The results indicate that 7% of the total variation in microbial community composition is explained by habitat ($R^2 = 0.07$), with a F-statistic of 2.5685, suggesting that between-habitat variation is approximately 2.57 times greater than within habitat variation. The resulting p-value (0.002) is highly significant.

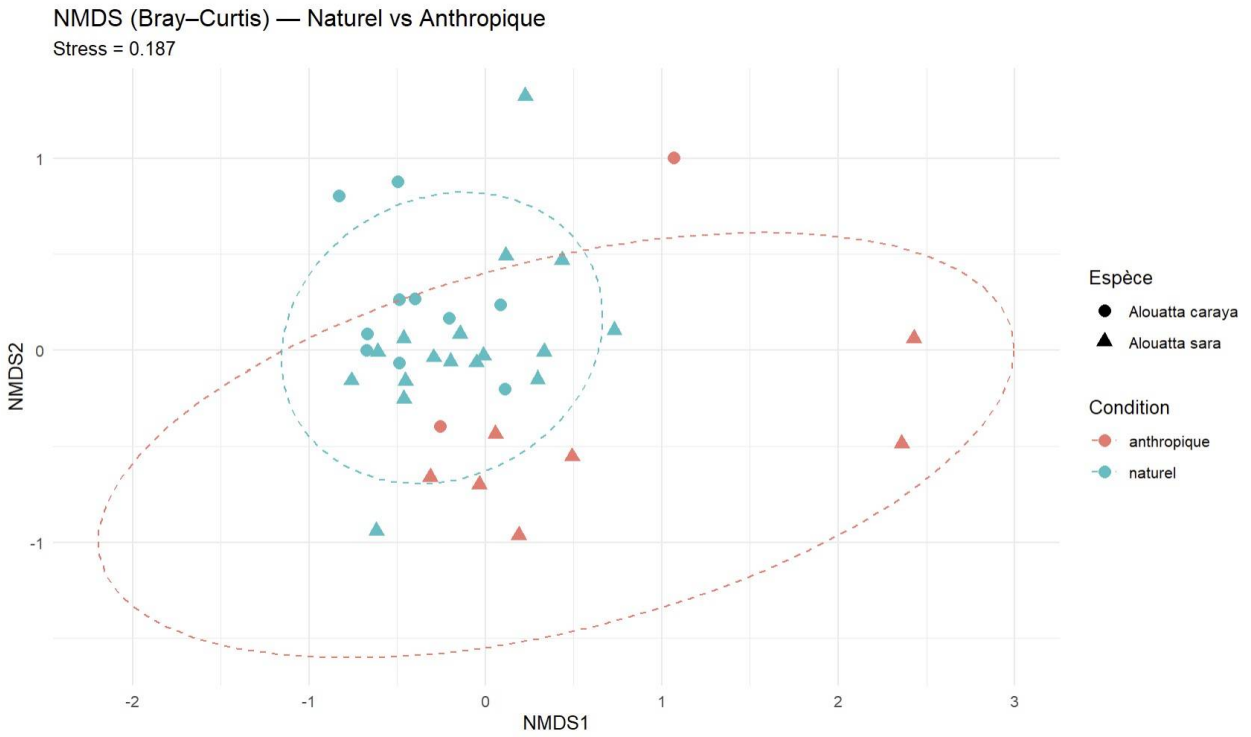

Figure 20. NMDS of the effect of habitat on microbiome composition

2. Influence of habitat on the microbiome in *Alouatta sara*

2.1. Alpha diversity

The Simpson index, showed slightly higher values in individuals A.sara from anthropogenic habitats compared to those from natural habitats (Figure 21).

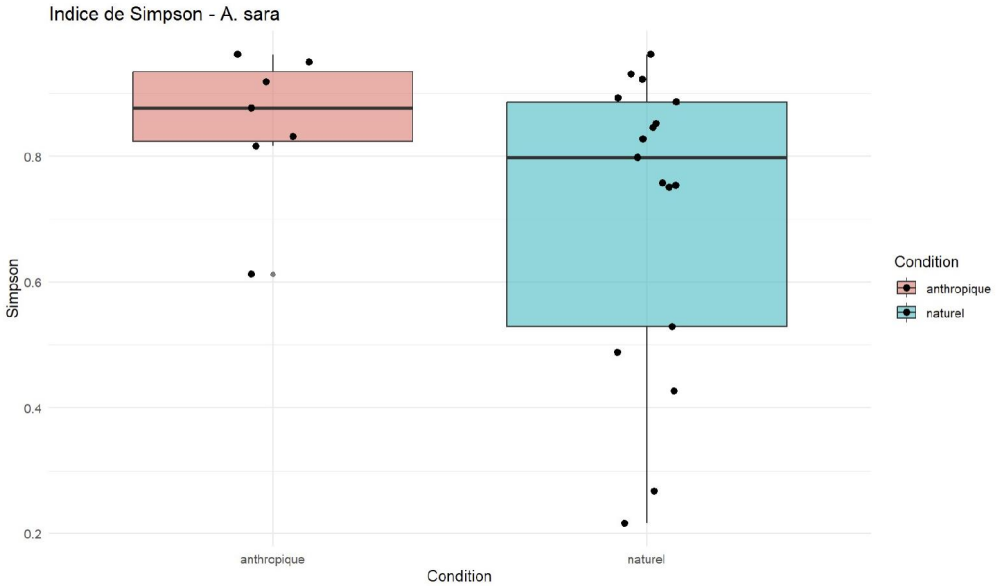

Figure 21. Simpson index in *Alouatta sara* by habitat type

However, the Shapiro-Wilk normality test showed that the distribution was not normal for the natural group ($p = 0.0101$), while it was normal for the anthropogenic group ($p = 0.1321$). As a result, a non-parametric comparison using the Wilcoxon-Mann-Whitney test was performed. The result ($p = 0.1662$) indicated that no significant difference in bacterial dominance was detected between the two habitat types. Regarding the Shannon index (Figure 22), which accounts for both

species richness and evenness, both groups displayed normally distributed values (Shapiro–Wilk: p = 0.4311 for the anthropogenic group and p = 0.7529 for the natural group). A Welch's t-test was

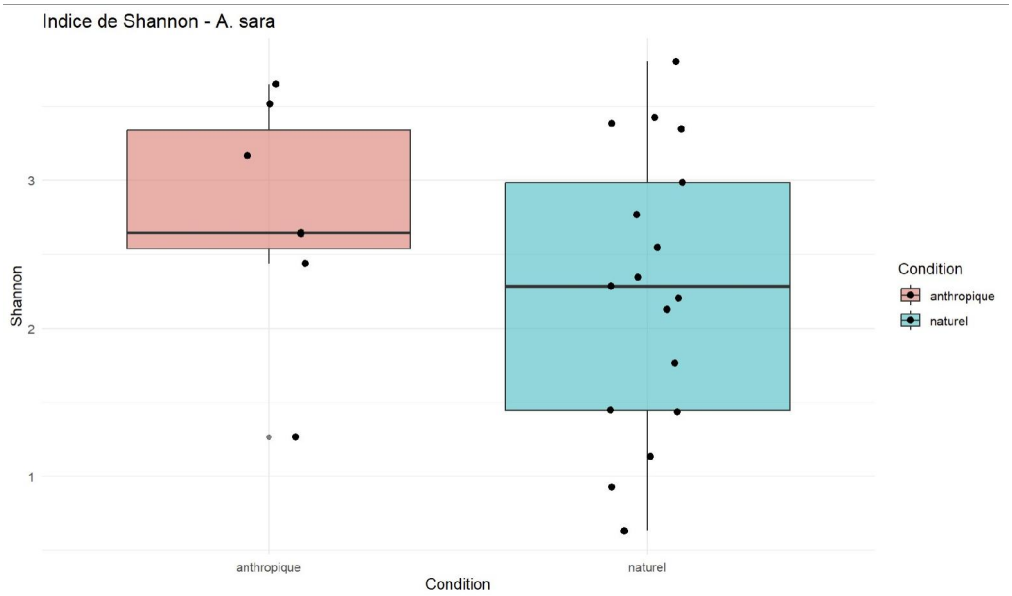

Figure 22. Shannon index in *Alouatta sara* by habitat type

therefore applied to avoid assuming equal variances, given the unequal sample sizes. The mean Shannon index was higher in the anthropogenic habitat (2.76) than in the natural habitat (2.27), but the difference was not statistically significant (p = 0.2201). The 95% confidence interval [-0.33 ; 1.31] included zero, confirming that this difference could be due to random variation.

2.2. Beta diversity

The observation of the Beta diversity made by PCoA and NMDS revealed partial clustering by habitat type. We can see a clear visual separation between individuals from anthropogenic and natural environments. The NMDS had a stress value of 0.147, indicating a satisfactory representation quality (stress < 0.2). In both ordination plots (Figure 23)(Figure 24), individuals from the two habitats showed some degree of overlap, yet distinct clustering patterns were also apparent.

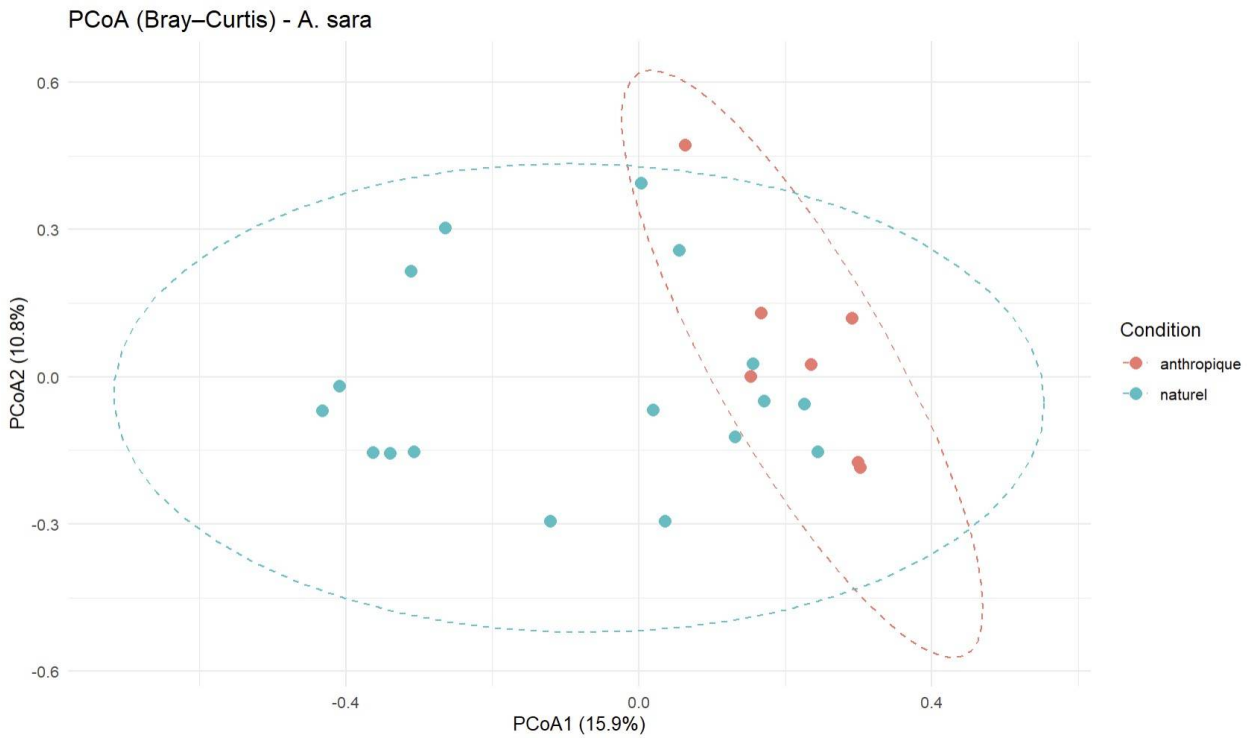

Figure 23. PCoA of the effect of habitat on microbiome composition in *Alouatta sara*

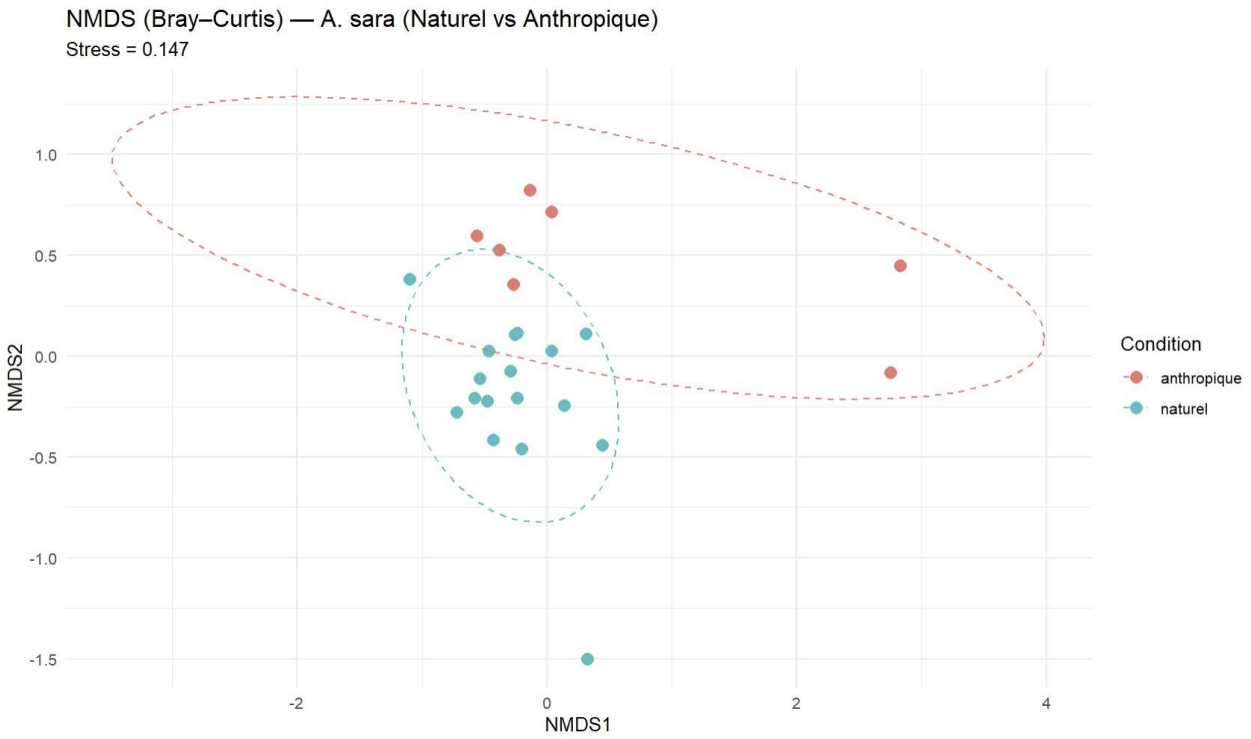

Figure 24. NMDS of the effect of habitat on microbiome composition in *Alouatta sara*

To statistically test this observation, a PERMANOVA was performed on the Bray-Curtis distance matrix. The results showed that habitat explains approximately 9.93% of the total variation in the microbiome ($R^2 = 0.0993$), with between-group variation being approximately 2.43 times greater than within-group variation ($F = 2.426$). The resulting p-value ($p = 0.001$) indicates that this difference is highly significant.

3. Influence of habitat on the microbiome in *Alouatta caraya*

3.1. Alpha diversity

The effect of habitat type (natural vs. anthropogenic) on the microbial diversity of *Alouatta caraya* was evaluated using the Shannon and Simpson indices (Annex - Figure S11 S12). However, only two out of four samples collected from anthropogenic environments could be analyzed, limiting the statistical power of the comparison. The Shannon index, which accounts for both species richness and evenness, showed mean values of 2.00 for the anthropogenic group and 2.21 for the natural group. The Simpson index, which emphasizes species dominance, had mean values of 0.73 and 0.77, respectively. Due to the limited number of samples from *A. caraya* in anthropogenic environments and the inability to properly test for normality, a non-parametric Wilcoxon test was applied for both indices. For the Shannon index, the resulting p-value ($p = 0.7576$) did not indicate any significant difference in diversity between habitats. Similarly, for the Simpson index, no significant difference was detected ($p > 0.05$).

3.2. Beta diversity

The Bray–Curtis distance matrix is visualized with a PCoA (PCoA) (Annexe - Figure S13) showing no clear clustering of samples based on habitat type was observed: individuals from natural and anthropogenic environments were distributed without a distinct separation. The PERMANOVA statistical analysis confirmed this lack of structuring, with a non-significant p-value ($p = 0.317$). These results indicate that no statistically significant differences were observed.

4. Influence of species (*A. sara* vs *A. caraya*) on the microbiome

The objective of this analysis was to determine whether microbiome diversity and composition differed significantly between the two studied species, *Alouatta sara* and *Alouatta caraya*, considering only individuals from natural habitats.

4.1. Alpha diversity

For the Simpson index (Figure 25), the Shapiro–Wilk test indicated a non-normal distribution for *A. sara* ($p = 0.01247$) and a normal distribution for *A. caraya* ($p = 0.3222$). Since the normality assumption was not met for both groups, a non-parametric Wilcoxon test was applied. This test revealed no significant difference between the two species ($p = 0.7956$), indicating that bacterial species dominance is comparable between *A. sara* and *A. caraya*. The two microbiomes therefore exhibit a similar balance, with no indication of one species being strongly dominated by a few taxa.

For the Shannon index (Figure 26), both distributions met the normality assumption (*A. sara*: $p = 0.8471$; *A. caraya*: $p = 0.1937$). A Student's t-test with Welch's correction was therefore applied, revealing no significant difference between the two species ($p = 0.7523$). The confidence interval, which includes zero, further confirms that the observed difference in means could be attributed to random variation.

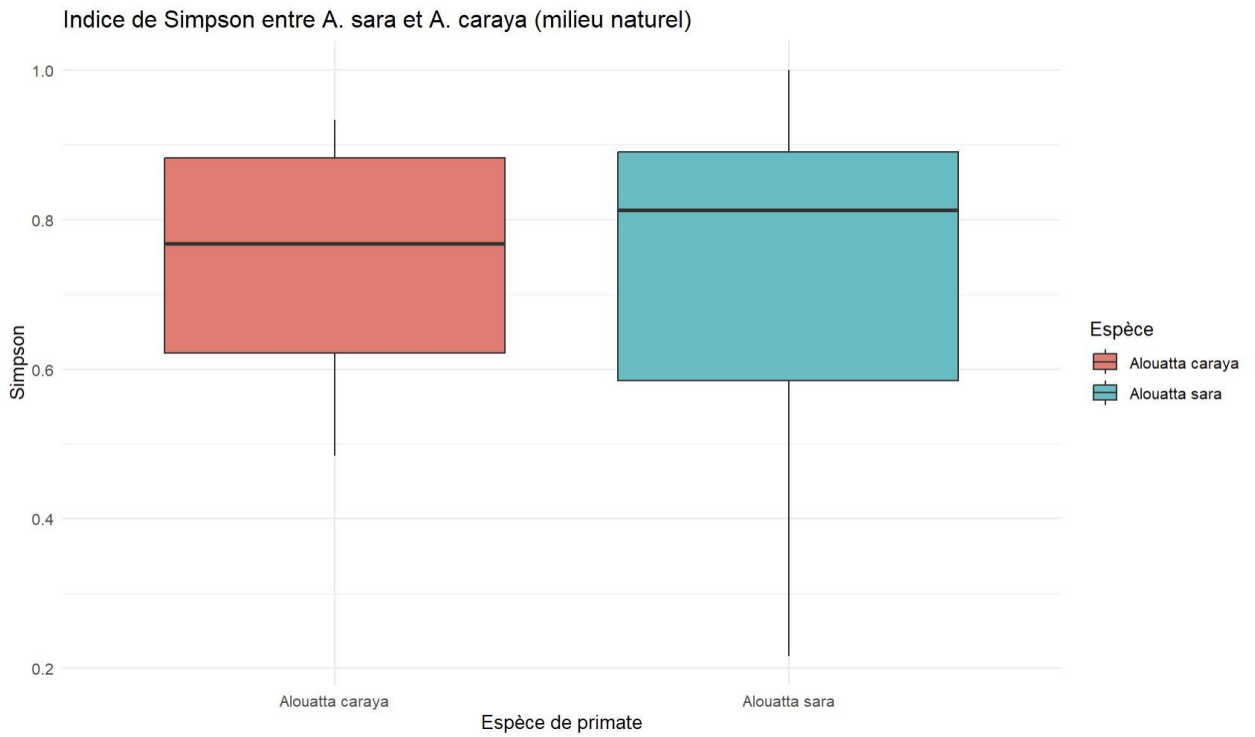

Figure 25. Simpson index comparing microbial dominance between *Alouatta sara* and *Alouatta caraya* in natural habitats

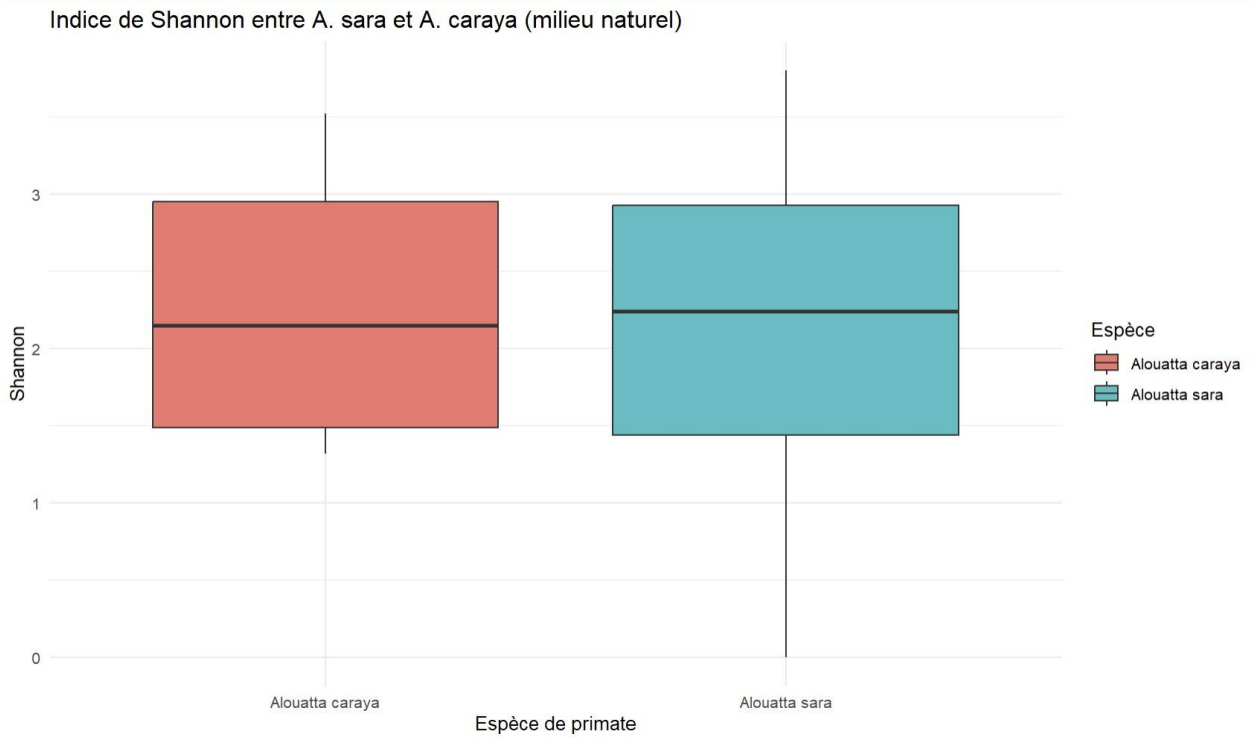

Figure 26. Shannon index comparing microbial dominance between *Alouatta sara* and *Alouatta caraya* in natural habitats

4.2. Beta diversity

The bray-curtis distance matrix is visualized using a PCoA (Figure 27), and a PERMANOVA test was performed to statistically assess the effect of species on microbial community composition. The PCoA visualization shows a degree of overlap between the two groups with a modest separation. PERMANOVA revealed a significant but weak effect of species ($p = 0.021$), with only 4.73% of the total variation in microbial composition explained ($R^2 = 0.04732$). The F-statistic

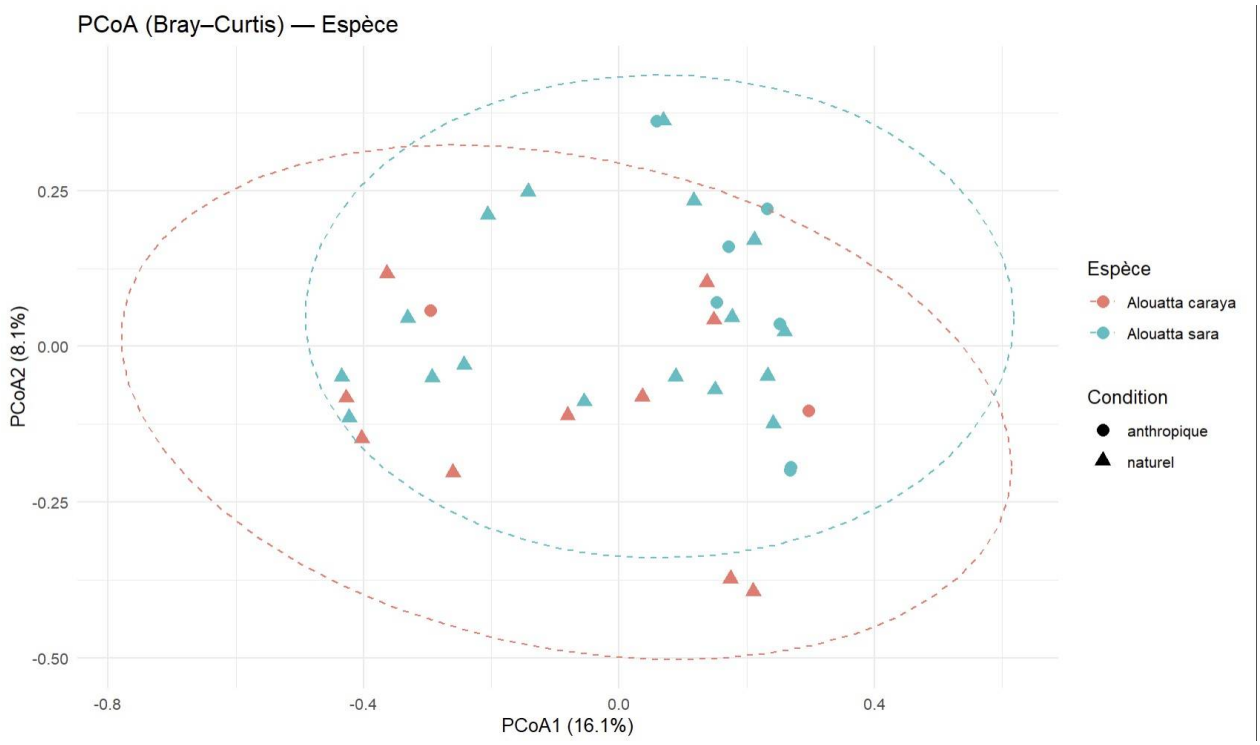

Figure 27. PCoA of the effect of species on microbiome

($F = 1.6886$) indicates that between-species variation is approximately 1.7 times greater than within-species variation, reflecting a moderate separation of the microbiomes.

5. Combined influence of species and habitat on the microbiome

The PCoA (Figure 28) allows to show the distribution of samples with respect to both factors (species x habitat). The ordination plot shows some overlap between groups, but also reveals trends toward separation based on species x habitat combinations. To statistically test this ob-

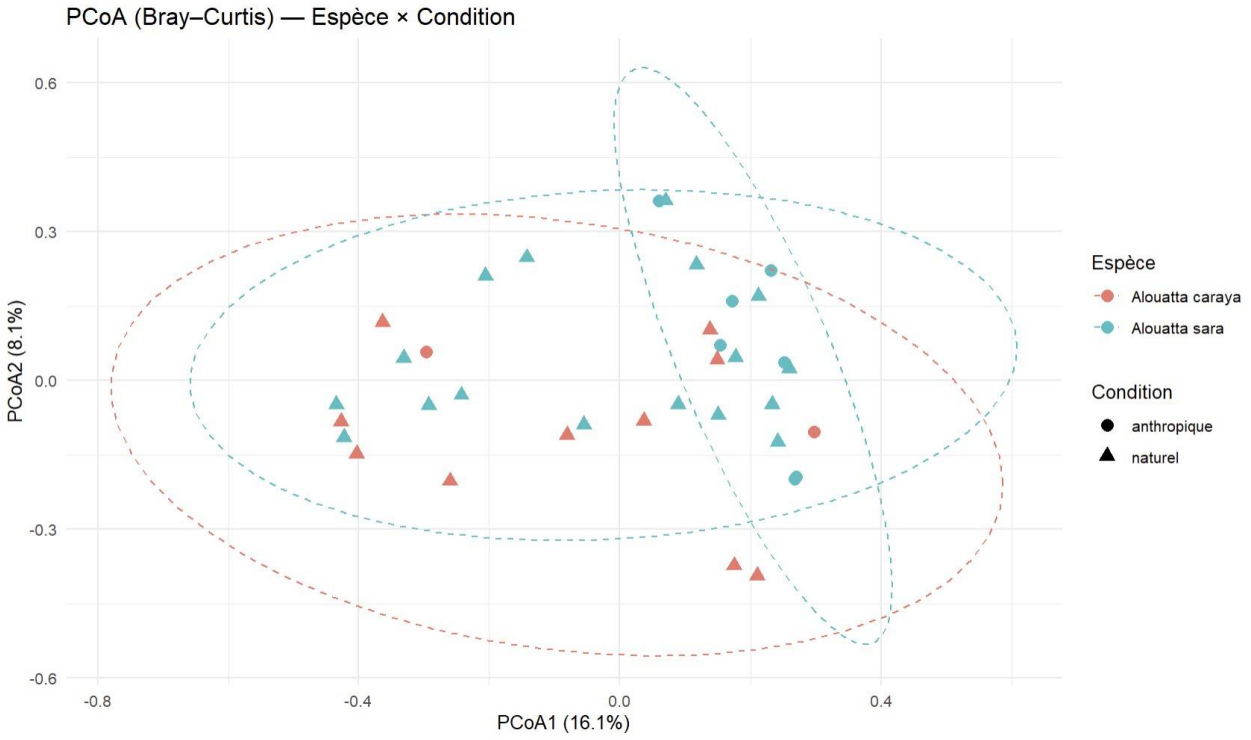

Figure 28. PCoA of the combined effects of species and habitat on the microbiome

servation, a PERMANOVA was performed on the Bray-Curtis distance matrix, including species, habitat, and their interaction as explanatory factors. The results indicate that the combined effect of species and habitat explains 14.3% of the total variation in microbial composition ($R^2 = 0.14288$), with an F-statistic of 1.7781. This F-value indicates that between-group variability is approximately 1.78 times greater than within-group variability, reflecting a moderate separation between species $\times$ habitat combinations. The associated p-value ($p = 0.001$) is highly significant.

6. Descriptive analysis of differences in bacterial composition between natural and anthropogenic habitats

Although alpha diversity indices (Shannon and Simpson) did not reveal significant differences between natural and anthropogenic habitats, this does not imply that bacterial communities are similar. Indeed, the graphical representations reveal a marked variation in community composition, with a higher relative abundance of the classes Actinobacteria and Bacilli in samples from anthropized environments, whereas samples from natural environments exhibit a higher relative abundance of the classes Clostridia, Bacteroidia, and Gammaproteobacteria (Figure 29).

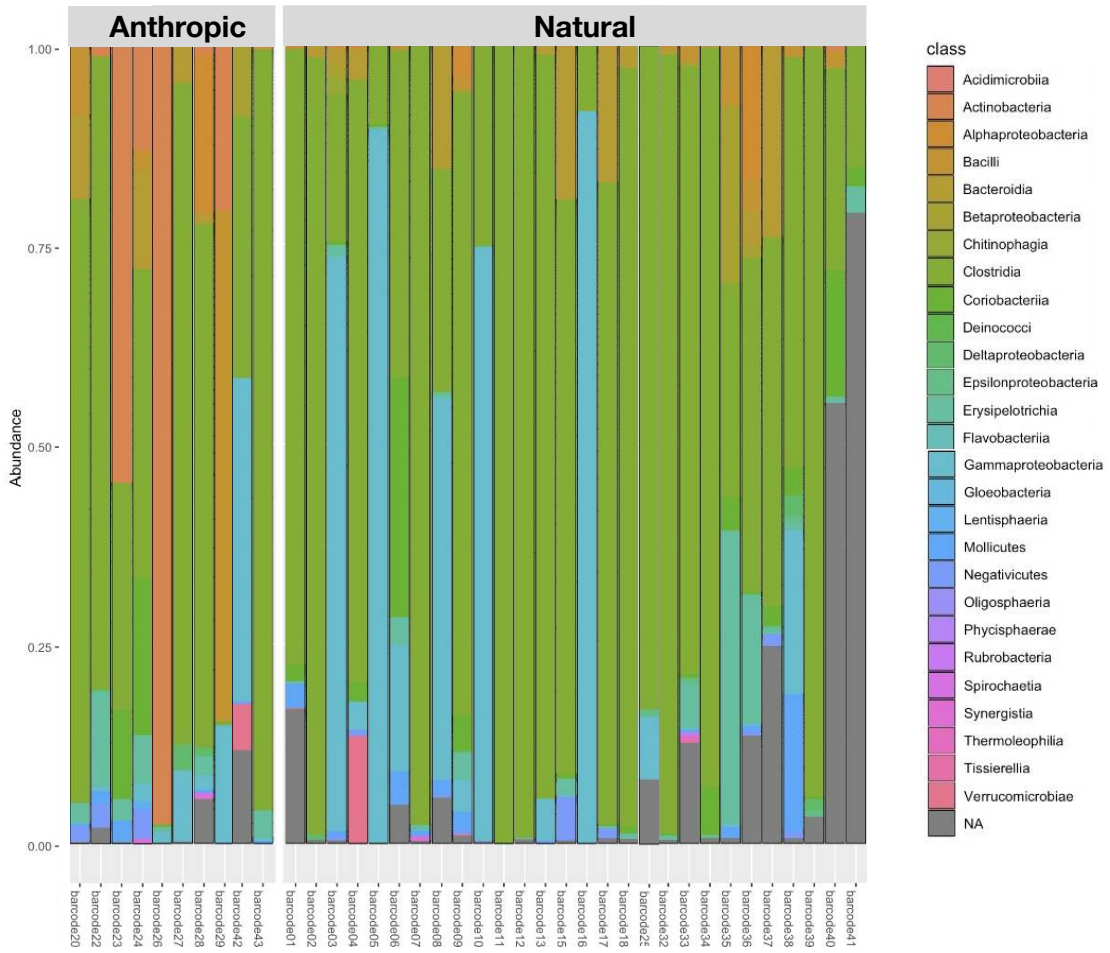

Figure 29. Relative abundance of bacterial classes in gut microbiomes of individuals from anthropic vs. natural habitats. Each barcode (column) represents one individual. NA = not assigned

Figure 30, and 31 shows a pronounced difference in the bacterial abundance of species belonging to the class Actinobacteria between natural and anthropogenic habitats.

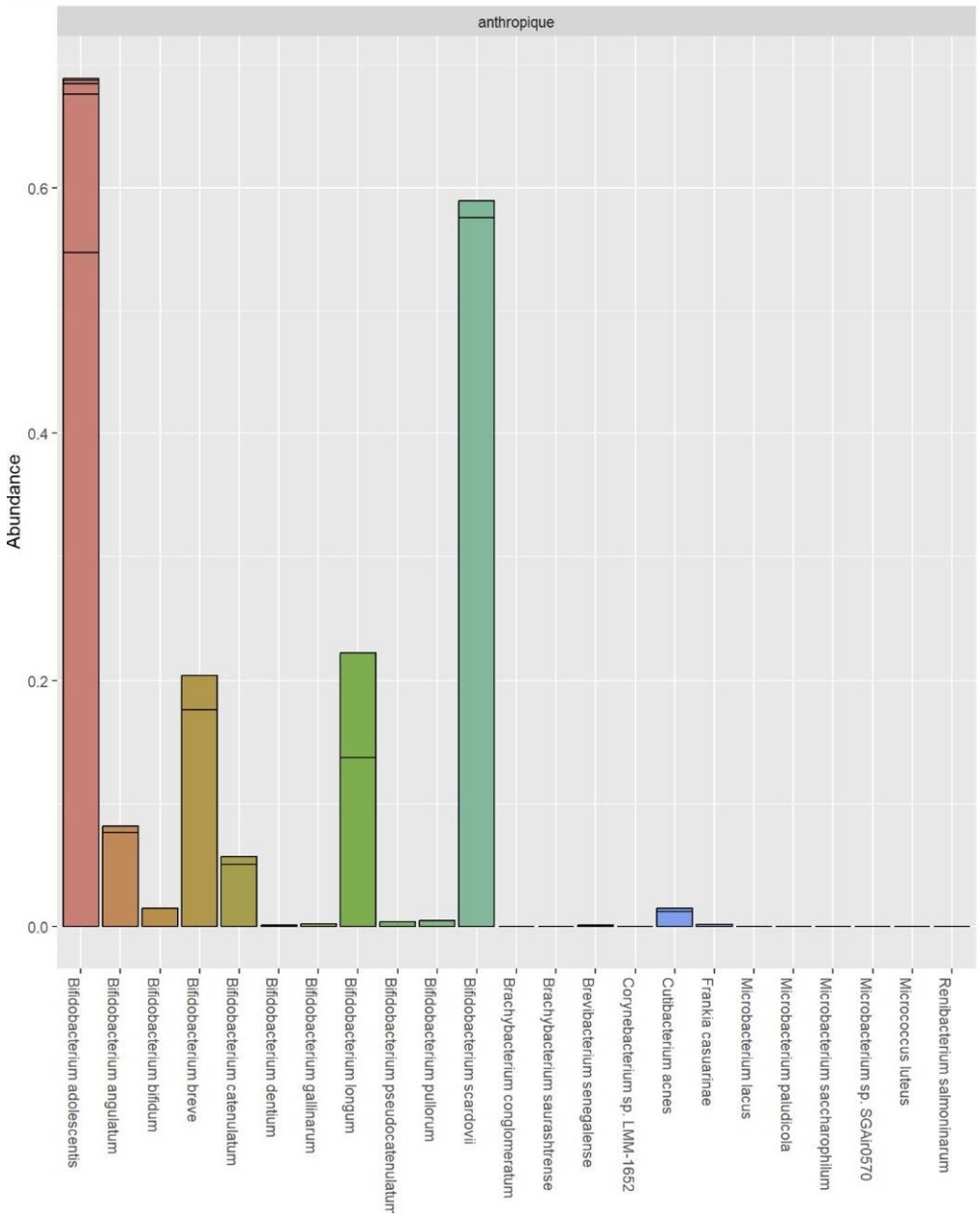

Figure 30. Abundance of different bacterial species within the class Actinobacteria across anthropogenic environments

Figure 32, which depicts the abundance of bacterial species belonging to the class Bacilli, also reveals differences in bacterial abundance between natural and anthropogenic habitats.

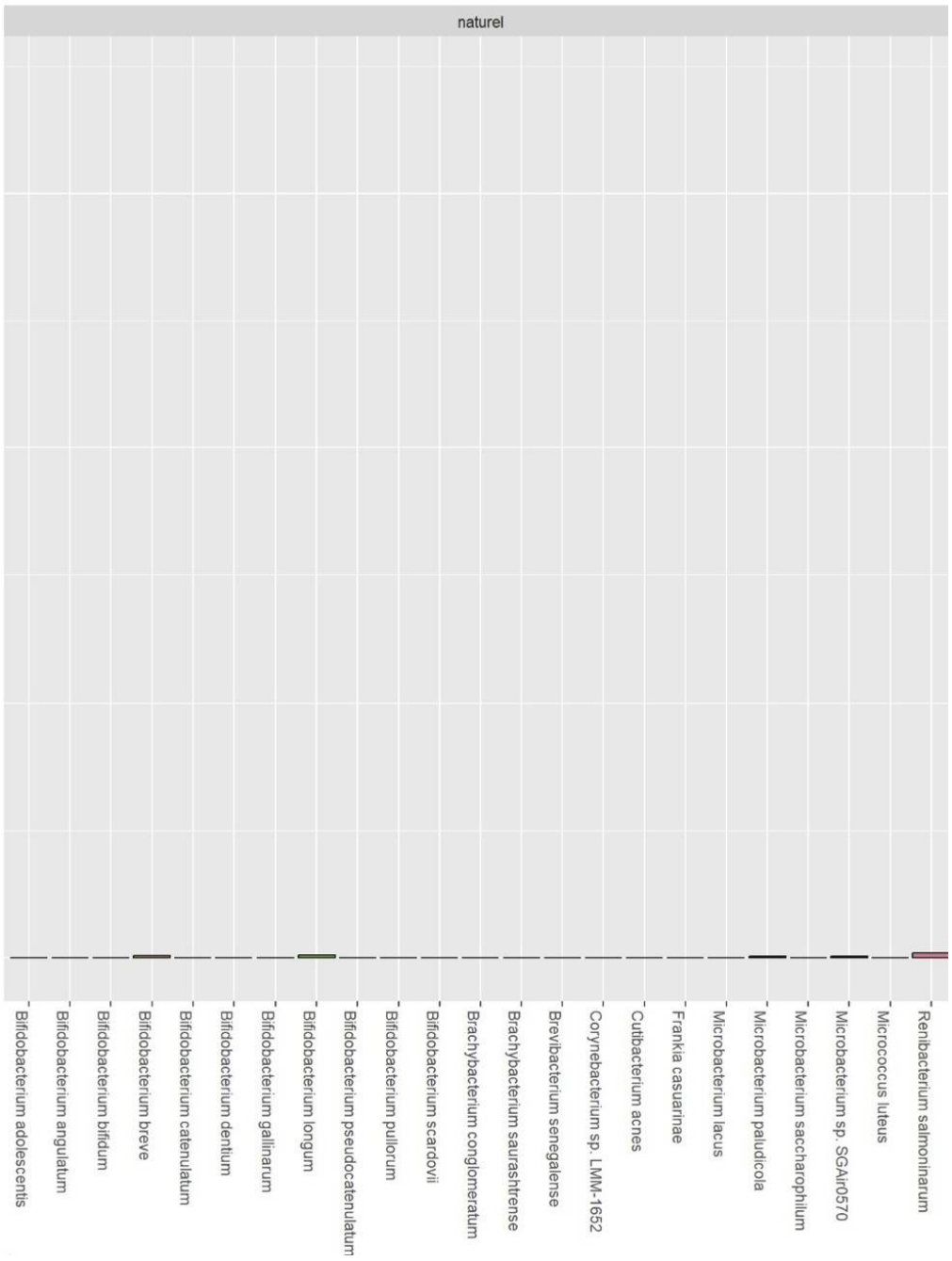

Figure 31. Abundance of different bacterial species within the class Actinobacteria across natural

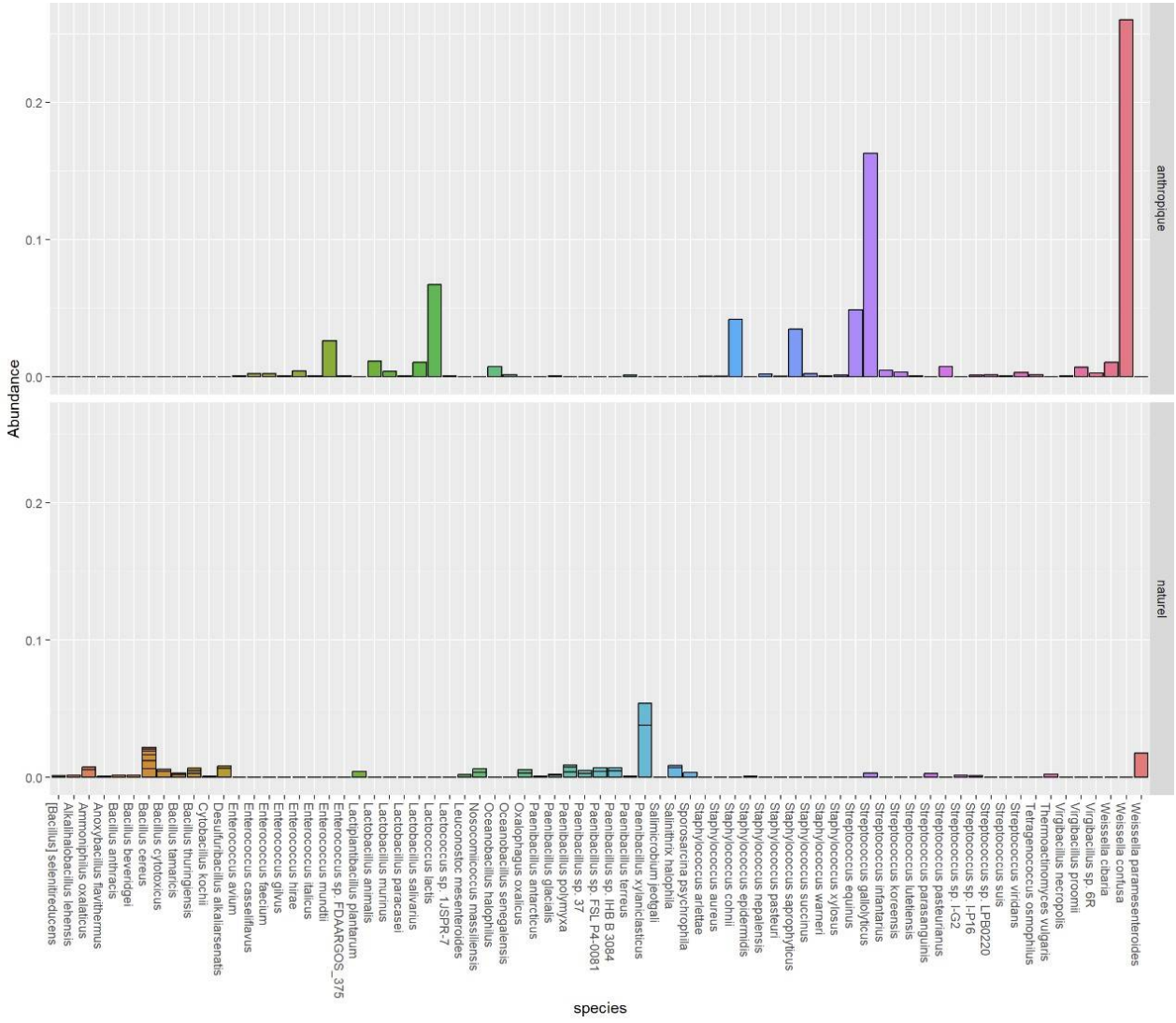

Figure 32. Abundance of different bacterial species within the class Bacilli across natural and anthropogenic environments.

7. Haplotype network

As previously mentioned, two molecular markers were used to identify species, the Internal Transcribed Spacer 2 (ITS2) region of nuclear ribosomal DNA and the cytochrome *b* gene (*cytb*) located in the mitochondrial genome. Unlike the primers designed to amplify the *cytb* gene, the ITS primers were not species-specific, resulting in non-target amplification. Consequently, the ITS sequences obtained corresponded to various plant species ingested by the primates, and could not be used for species identification. In contrast, *cytb* sequencing proved successful. The sequences generated were compared to the NCBI database (Sayers et al., 2025) using the Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990). These comparisons unambiguously confirmed species identity. Individuals identified as *Alouatta sara* on the field matched known *A. sara* sequences, and the same was true for *A. caraya*. To further support these results, a haplotype network was constructed using HaplowebMaker (Spöri and Flot, 2020) based on *cytb* sequences (Figure 33). This type of representation allows visualization of sequence differences (mutations) between haplotypes and their evolutionary proximity. Each circle represents a unique haplotype (i.e. a distinct sequence), with its size proportional to the number of individuals sharing it. Grey lines between haplotypes indicate evolutionary connections, while black bars represent the number of nucleotide substitutions separating the sequences.

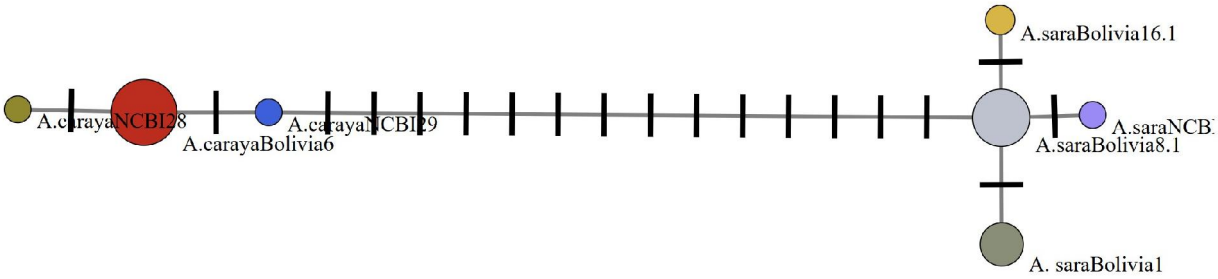

Figure 33. Haplotype network of A. caraya and A. sara sequences.

In Figure 33, The haplotypes labeled "A.carayaBolivia" represent sequences directly generated from my own field samples collected in Bolivia, while "A.carayaNCBI" refer to sequences retrieved from the NCBI database (Sayers et al., 2025) ; the same naming convention applies for *A. sara*.

Discussion

During the fieldwork for this study, several discoveries were made. I discovered new contact zones that had not been reported until now. As shown in (Figure 34), the theoretical distribution zones of the two species are displayed to date. However, at field site n°5, I observed the presence of *Alouatta caraya* as well as *Alouatta sara* to the east of the Río Grande in the municipality of San Antonio de Lomerío. There is thus at least one new contact zone between the two species living sympatrically beyond the Río Grande, to the east of the department of Santa Cruz de la Sierra, in the Ñuflo de Chávez province, in the municipality of San Antonio de Lomerío (Figure 34). The observation took place in a private area belonging to the San Miguelito Jaguar Conservation Ranch at coordinates 17°01.676'N 061°55.661'W. On the other hand, the distribution zone of *Alouatta caraya* should be reduced, as in several localities to the west of Santa Cruz de la Sierra (e.g., Buena Vista, Rosa del Sara, or Amboró National Park), *Alouatta caraya* is absent (Figure 34). Within the new contact zone discovered at the San Miguelito Jaguar Conservation Ranch (17°1'38.72"S, 61°51'52.01"W), a mixed group of *Alouatta sara* and *Alouatta caraya* was observed. The alpha male of the group exhibited a hybrid phenotype. His coloration pattern was an intermediate between the dark black coloration of *A. caraya* males and the red coloration of *A. sara* individuals, strongly suggesting hybridization between the two species (Figure 35). Natural hybridization in primates can be an important evolutionary mechanism (Arnold and Meyer, 2006), and cases of hybridization between other howler monkey species (*A. caraya* and *A. clamitans* in southern Brazil, and *A. palliata* and *A. pigra* in Mexico) have already been recorded (Aguiar et al., 2008). Therefore, it would be interesting to conduct more specific studies on this potential hybridization phenomenon, as well as on the likelihood of genetic introgression between the species that may follow (Mourthe et al., 2019)

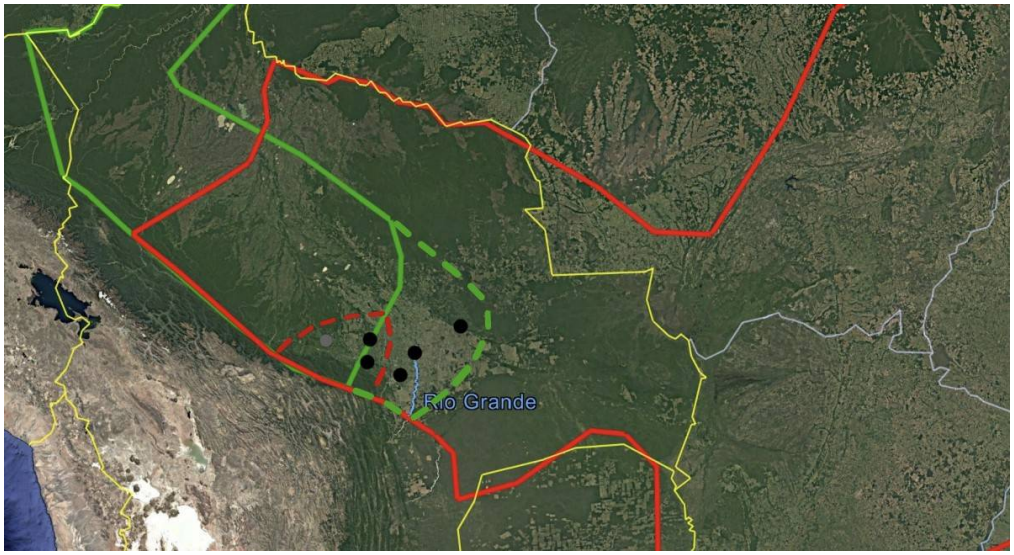

Figure 34. Proposed new geographic distribution for A. sara (in green) and A. caraya (in red). Dashed lines indicate the newly defined area for each species. Black dots represent sampling sites, and the gray dot represents an observed site.

The selection of sampling sites was crucial to ensure the collection of a sufficient number of samples, enabling the continuity of the research. Second, a robust sampling protocol was developed to minimize contamination from field collection to sample repatriation. The protocol adopted, combined with the sampling tubes provided by the Environmental Dropping Sampling Kit from Sylphium® (ref. SYL006), offered substantial operational flexibility. This system allows for the preservation of fecal samples without immersion in ethanol or immediate freezing, thereby removing many logistical constraints in the field.

The selected sampling sites proved adequate; however, better planning in advance, along with

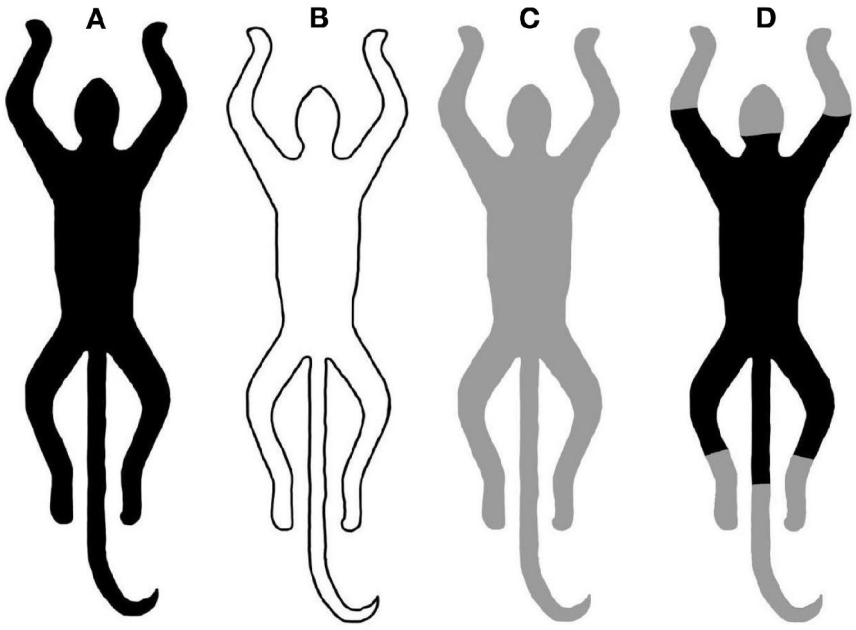

Figure 35. Phenotype of *A. caraya*, *A. sara*, and hybrid phenotype.
A – *A. caraya* black male. B – *A. caraya* yellowish-brown female. C – *A. sara* reddish coat. D – A Hybrid coat between *A. sara* and *A. caraya*

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more frequent communication with local collaborators, could optimize on-site time management. Due to the time constraints imposed by the schedule of this thesis, the field mission was conducted during the wet season, rendering the "Ibbecken" site (Table 1) inaccessible. Sampling during the dry season would have facilitated site accessibility and improved visibility of individuals through the canopy.

The mission itself allowed the acquisition of new skills, gained both through prior research and the assistance of local collaborators. Nevertheless, the unpredictability of the studied individuals suggests that extending the sampling duration at each site would help secure a sufficient number of samples.

Once the samples were brought back to Belgium, laboratory analyses could begin. DNA extraction was performed using the kit provided by the same company that supplied the collection tubes, Sylphium® (ref. SYL002/100/000, lot 240130). However, this kit presented significant technical challenges throughout the project. Given that fecal samples are often rich in PCR inhibitors, it is essential to use a kit capable of both efficient extraction and thorough purification. In this case, residual impurities in the DNA extracts resulted in the inhibition of numerous PCR reactions. Several rounds of testing were required to evaluate the impact of final DNA concentrations and 260/230 and 260/280 absorbance ratios on amplification success. For some samples, no PCR amplification was successful.

To confirm the source of these issues, a second kit, identified later, was tested in the context of a secondary project. Fecal samples collected in Brazil by my supervisor were processed using the same protocol as in the present study. With the Sylphium® kit, only 7 out of 13 PCR reactions were successful. The protocol was then repeated using the DNeasy PowerSoil Pro Kit (Qiagen®), following the manufacturer's instructions. The results were unambiguous: all 13 PCR reactions produced successful amplifications (Annex 3 - S14). These findings indicate that replacing the Sylphium® kit with the Qiagen® kit in the initial protocol would likely improve both the yield and reliability of extractions, enabling sequencing from all collected samples and thus increasing both

the quantity and quality of available data. Moreover, such a change would considerably reduce the time required for laboratory manipulations, making the analyses more efficient and reproducible.

Regarding alpha diversity analyses, the null hypothesis (H0), stating no significant difference between the two habitat types or between the two species, could not be rejected based on the analyzed samples. This holds true both when comparing alpha diversity across habitat types (anthropogenic vs. natural) and when comparing alpha diversity between species. The results suggest that the two species share a similar overall bacterial diversity (in terms of richness and evenness) in their natural habitat, as well as when living either in captivity or in the wild. However, a comparison of bacterial composition between natural and anthropogenic habitats revealed clear differences in the taxa represented (Figures 29). The three phyla generally dominant in the gut microbiome of non-human primates, Firmicutes, Proteobacteria, and Bacteroidota (Clayton et al., 2018),were present in both environments, but with markedly different distributions (Figures 29). Actinobacteria (Figure 30 and 31), although naturally present in the gut microbiota, typically represent a minority component of the microbial community (Binda et al., 2018; Barka et al., 2015). However, several species of Actinobacteria found here, notably Bifidobacterium, are well-known for their use as probiotics (Mahalanobish et al., 2019). For instance, Bifidobacterium adolescentis, B. bifidum, B. breve, and B. longum are commonly used in probiotic formulations, often added to dietary supplements and fermented dairy products (Marteau et al., 2002; Duranti et al., 2020; Roberts et al., 2020). Their high abundance in anthropogenic samples may thus reflect either the intentional administration of probiotics in zoos or rescue centers, or cross-contamination with humans via food, veterinary care, or direct contact. Similarly, Bacilli (Figure 32), particularly the genera Streptococcus, Lactococcus, Enterococcus, and Weissella, are associated not only with the gut microbiota, but also with human skin, fermented food products, and probiotic formulations (Marteau et al., 2002; Vera Pingitore et al., 2012; Araújo and Ferreira, 2013; Fusco et al., 2015). In contrast, natural habitats exhibit a higher diversity of environmental bacterial species, notably taxa belonging to the genus Paenibacillus, which are known for their role in plant fiber degradation, their presence in soil, and their interactions with the gut microbiota of herbivores (Lal and Tabacchioni, 2009; Grady et al., 2016; McSpadden Gardener, 2004).Similarly, bacteria of the genus Bacillus, commonly found in soil, plants, and insects, were also more frequently detected in natural environments (McSpadden Gardener, 2004; Vilas-Bôas et al., 2007). Regarding the class Gammaproteobacteria (Figures 29), these bacteria are among the dominant classes in the gut microbiome of primates in natural environments (Clayton et al., 2018). However, their abundance was considerably lower in individuals living in anthropogenic habitats, where they were replaced by other bacterial classes. These observations suggest that captive individuals do not necessarily exhibit lower bacterial diversity, but that their microbiome composition is shaped by anthropogenic factors such as diet, supplements, probiotics, or direct contact with humans. In *Alouatta sara* and *Alouatta caraya*, certain bacterial taxa typical of natural habitats may therefore be replaced by human-associated species, maintaining overall diversity but altering its composition.

Beta diversity analyses, using PERMANOVA tests, indicated that the null hypothesis (H0), no significant difference in microbiome composition, could be rejected in most cases, except for the effect of habitat on *A. caraya*, likely due to insufficient sample size. The habitat factor (natural vs. anthropogenic) exerted the strongest influence (Figures 20, 23, and 24), followed by the species factor (Figure 27). The greatest differences in microbiome composition were observed when both factors were considered together (Figure 28). These results suggest that habitat and species interact to shape the bacterial community structure. A longitudinal study of *Alouatta* populations in both natural and captive environments, combined with controlled dietary modifications in captivity, could allow real-time assessment of these factors on microbial diversity.

Thanks to the haplotype network, Figure 33, we can observe two clearly distinct clusters. On the left, haplotypes associated with *Alouatta caraya*, centered around a frequent (purple) haplotype,

surrounded by a few closely related variants-indicative of low intra-specific divergence. On the right, haplotypes associated with *Alouatta sara*, similarly structured around a frequent (green) haplotype with short links to slightly divergent variants. A long series of mutations separates the two clusters, reflecting a clear genetic divergence, thereby confirming that the two species form distinct genetic entities. This analysis validates the assignment of my sequences to either *Alouatta sara* or *Alouatta caraya*

Conclusions and Perspectives

The results obtained in this study provide valuable insights into the research question: How does anthropogenic impact affect the microbiome in howler monkeys (genus *Alouatta*) in Bolivia? The data suggest that, in Bolivian howler monkeys (genus *Alouatta*), anthropogenic impact significantly influences the overall composition of the microbiome (beta diversity) but not its internal diversity (alpha diversity). Anthropogenic habitats are characterized by shifts in the classes and species of bacteria present in fecal samples, reflecting a reduced abundance of certain taxa typical of natural environments. This reduction appears to be compensated by the acquisition of new bacterial species, likely introduced through interactions with humans, such as direct contact, veterinary care, diet, and supplements. Furthermore, the results indicate that the primate species itself may exert a more moderate influence on microbiome composition.

This preliminary study has generated new knowledge on the structure and variability of the microbiome in the genus Alouatta and has provided a clearer understanding of the extent of changes induced by human interaction. From an institutional and methodological perspective, this work marks the starting point of a broader project aimed at exploring microbiome diversity in non-human primates. It has initiated a collaboration between the Université libre de Bruxelles (ULB) and the Noel Kempff Mercado Natural History Museum, establishing the foundations for a new line of research within the Ecological and Evolutionary Genomics laboratory. In addition, it has resulted in the development of a robust, non-invasive fecal sampling protocol for Alouatta, which could be readily adapted to other primate species.

Looking ahead, the continuation of this project as part of a doctoral thesis could allow for deeper investigation and broader taxonomic coverage, including an expanded range of species and an in-depth analysis of potential hybridization between A. sara and A. caraya. Such studies would strengthen our understanding of the effects of anthropogenic influence on primate health and microbial ecology, while contributing to the development of more targeted and effective conservation strategies.

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Annexes

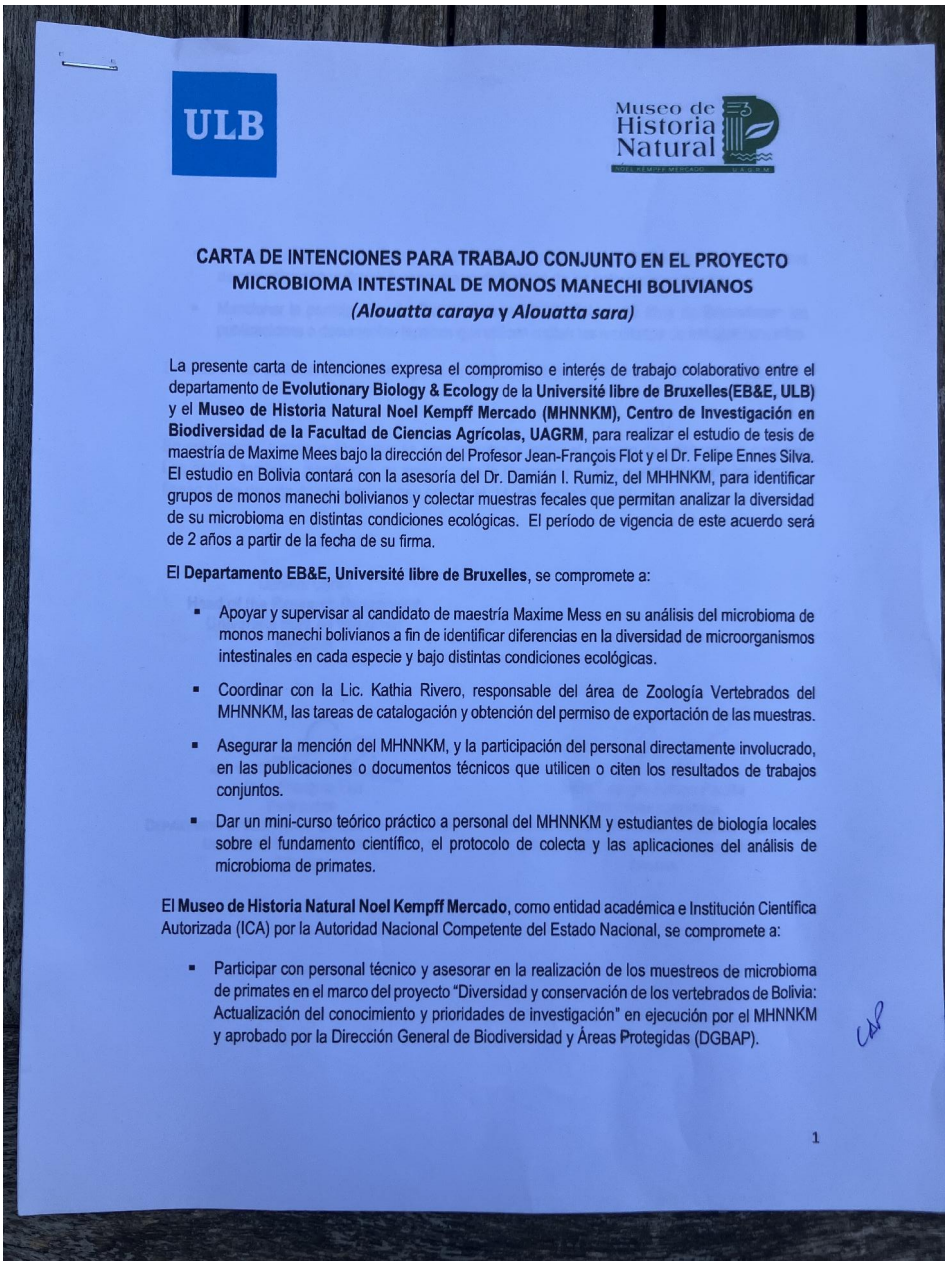
Annex 1: Official documents

ULB UNIVERSITÉ LIBRE DE BRUXELLES

SAMPLES COLLECTION FORM

Date: 28 / 03 /2024	Field code: MM20240328.1.01
Locality: Buena Vista (Bosque de José)	Time of Collection : 8h30
District or Municipality : département de Santa Cruz de La Sierra, province de Ichilo, municipalité de buena vista	Country : Bolivie
Coordinates: Lat: 17° 29. 972' Long: 063° 39. 061'	
Species: Alouatta sara	
(X) Male ♂	( ) Female ♀
Age category: ( ) Infant ( ) Juvenil ( ) Subadult (X) Adult	
GROUP INFORMATION	
Mix pattern (*A. sara* X *A. caraya*) of pelage colouration? ( ) Yes (X) No	
Number of individuals sighted: 8	
Number of Adult Males 1	
Number of Adult Females 2	
Number of immatures (infant, juveniles and subsdults) 2 infant (bébé), 3 juvenile	
OTHER INFORMATION: 1 mâle adulte, 2 femelles adultes, 2 enfants (bébé encore accroché à leur mère ; cf. vidéo), 3 juvéniles. Nous avons observé un groupe de 11 individus l'autre jour, composé de 2 mâles adultes, 2 bébés, 1 enfant, 3 juvéniles et 3 femelles adultes. Le jour de la collecte, un groupe de 8 individus était présent, dont 7 échantillons différents ont été collectés. Il manquait 1 mâle, 1 femelle et 1 juvénile. Ces derniers ont été observés à 231 m au sud-est du lieu de collecte, avec un individu supplémentaire (juvénile) près de la maison de José. José a déjà observé un mâle adulte seul se promenant dans la forêt. On pense donc que le groupe est en train de se séparer en deux groupes distincts, car il y a trop de mâles adultes dans le groupe initial. Coordonnées du petit groupe en formation potentielle : Lat. (S) : 17° 29.884' Long. (O) : 063° 39.134'	

Figure S1. Field data sheet of the sample n°1



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Museo de Historia Natural

CARTA DE INTENCIONES PARA TRABAJO CONJUNTO EN EL PROYECTO MICROBIOMA INTESTINAL DE MONOS MANECHI BOLIVIANOS
(Alouatta caraya* y *Alouatta sara)

La presente carta de intenciones expresa el compromiso e interés de trabajo colaborativo entre el departamento de **Evolutionary Biology & Ecology** de la **Université libre de Bruxelles(EB&E, ULB)** y el **Museo de Historia Natural Noel Kempff Mercado (MHNNKM), Centro de Investigación en Biodiversidad de la Facultad de Ciencias Agrícolas, UAGRM**, para realizar el estudio de tesis de maestría de Maxime Mees bajo la dirección del Profesor Jean-François Flot y el Dr. Felipe Ennes Silva. El estudio en Bolivia contará con la asesoría del Dr. Damián I. Rumiz, del MHHNKM, para identificar grupos de monos manechi bolivianos y colectar muestras fecales que permitan analizar la diversidad de su microbioma en distintas condiciones ecológicas. El período de vigencia de este acuerdo será de 2 años a partir de la fecha de su firma.

El **Departamento EB&E, Université libre de Bruxelles**, se compromete a:

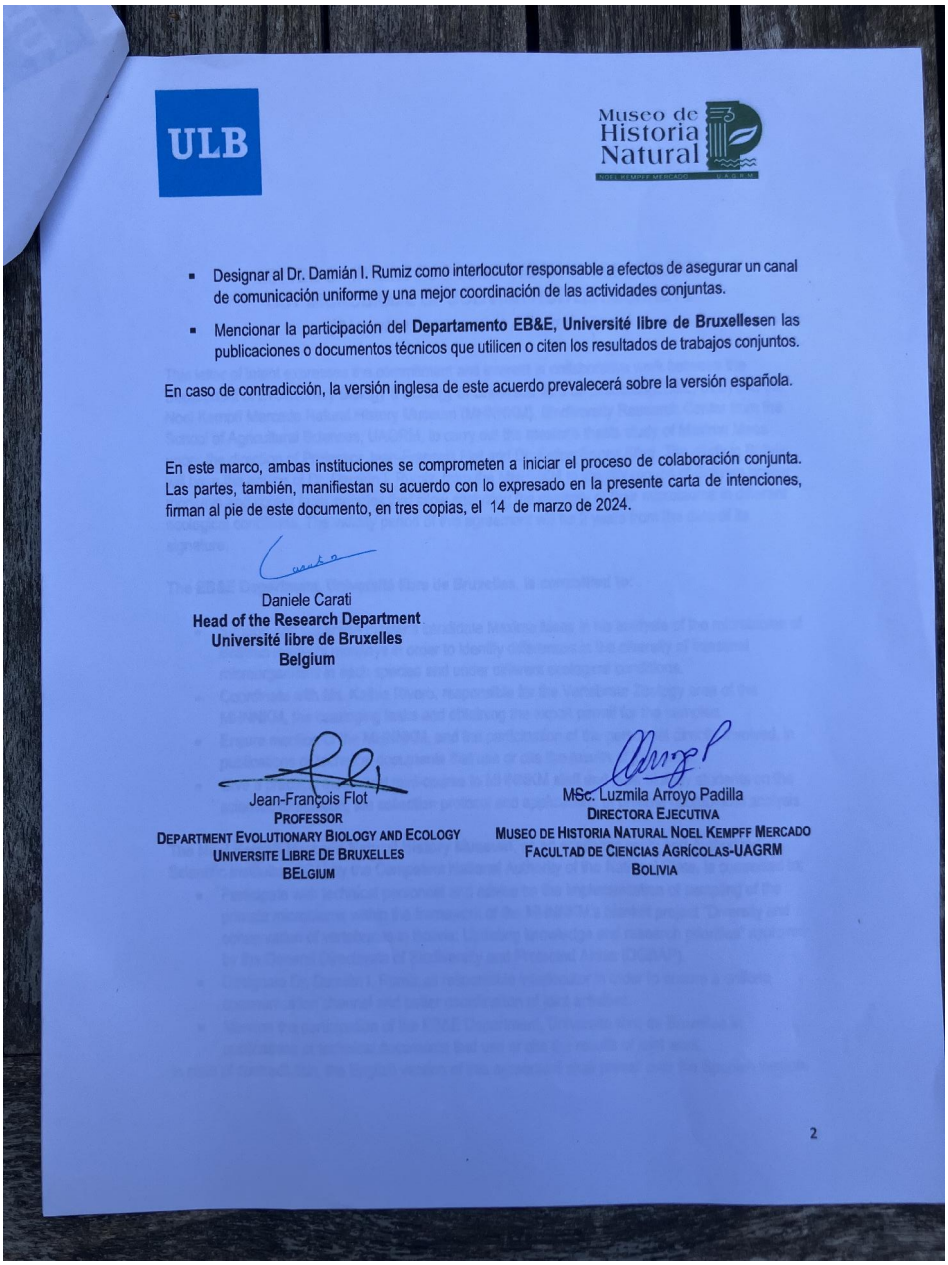
- Apoyar y supervisar al candidato de maestría Maxime Mess en su análisis del microbioma de monos manechi bolivianos a fin de identificar diferencias en la diversidad de microorganismos intestinales en cada especie y bajo distintas condiciones ecológicas.
- Coordinar con la Lic. Kathia Rivero, responsable del área de Zoología Vertebrados del MHNNKM, las tareas de catalogación y obtención del permiso de exportación de las muestras.
- Asegurar la mención del MHNNKM, y la participación del personal directamente involucrado, en las publicaciones o documentos técnicos que utilicen o citen los resultados de trabajos conjuntos.
- Dar un mini-curso teórico práctico a personal del MHNNKM y estudiantes de biología locales sobre el fundamento científico, el protocolo de colecta y las aplicaciones del análisis de microbioma de primates.

El **Museo de Historia Natural Noel Kempff Mercado**, como entidad académica e Institución Científica Autorizada (ICA) por la Autoridad Nacional Competente del Estado Nacional, se compromete a:

- Participar con personal técnico y asesorar en la realización de los muestreos de microbioma de primates en el marco del proyecto "Diversidad y conservación de los vertebrados de Bolivia: Actualización del conocimiento y prioridades de investigación" en ejecución por el MHNNKM y aprobado por la Dirección General de Biodiversidad y Áreas Protegidas (DGBAP).

1

Figure S2. Letter of intent - Spanish version



- Designar al Dr. Damián I. Rumiz como interlocutor responsable a efectos de asegurar un canal de comunicación uniforme y una mejor coordinación de las actividades conjuntas.
- Mencionar la participación del **Departamento EB&E, Université libre de Bruxelles**en las publicaciones o documentos técnicos que utilicen o citen los resultados de trabajos conjuntos.

En caso de contradicción, la versión inglesa de este acuerdo prevalecerá sobre la versión española.

En este marco, ambas instituciones se comprometen a iniciar el proceso de colaboración conjunta. Las partes, también, manifiestan su acuerdo con lo expresado en la presente carta de intenciones, firman al pie de este documento, en tres copias, el 14 de marzo de 2024.

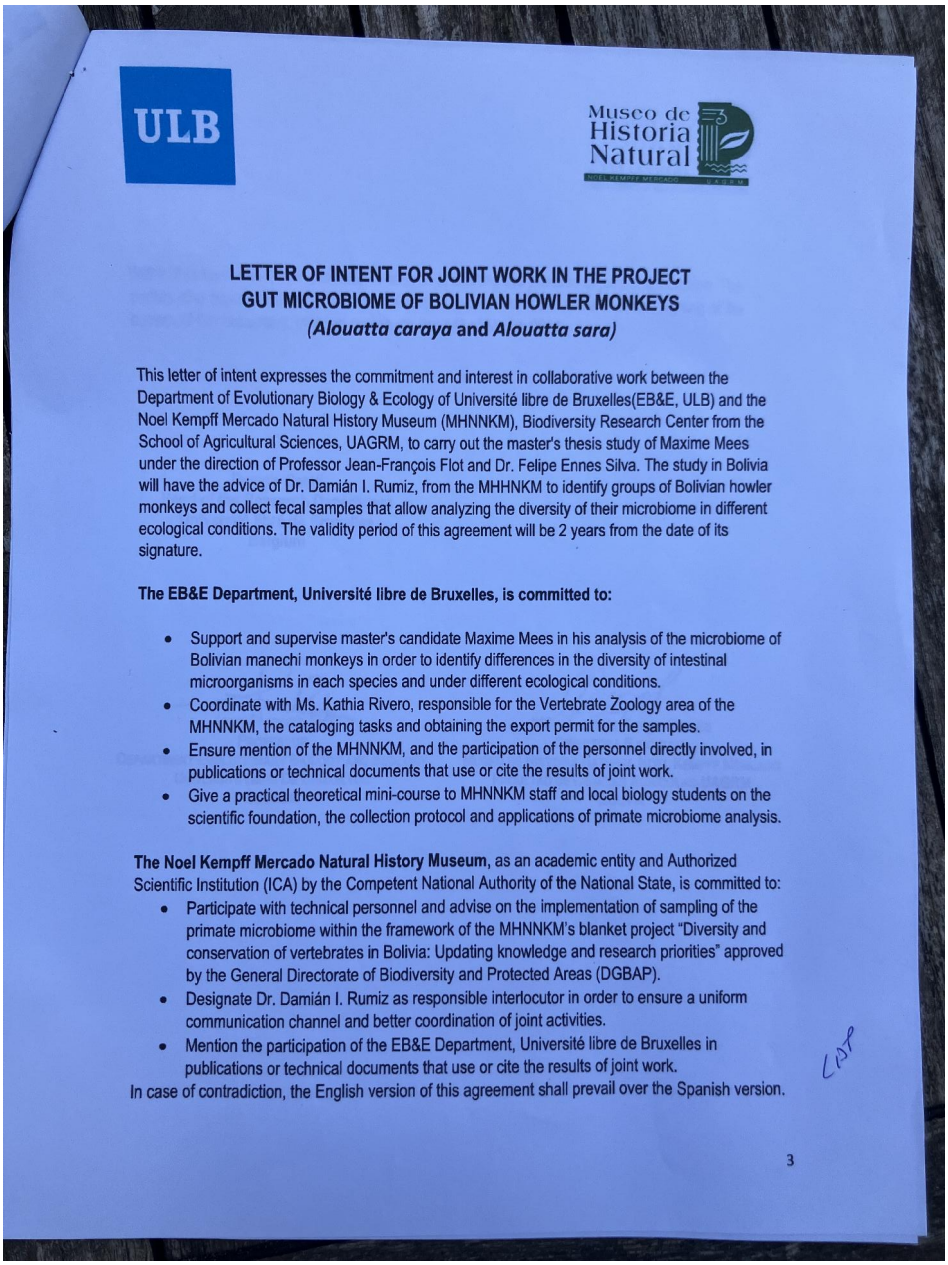
Daniele Carati
Head of the Research Department
Université libre de Bruxelles
Belgium

Jean-François Flot
PROFESSOR
DEPARTMENT EVOLUTIONARY BIOLOGY AND ECOLOGY
UNIVERSITE LIBRE DE BRUXELLES
BELGIUM

MSc. Luzmila Arroyo Padilla
DIRECTORA EJECUTIVA
MUSEO DE HISTORIA NATURAL NOEL KEMPFF MERCADO
FACULTAD DE CIENCIAS AGRÍCOLAS-UAGRM
BOLIVIA

2

Figure S3. Letter of intent - Spanish version



ULB

Museo de Historia Natural
NOEL KEMPFF MERCADO

LETTER OF INTENT FOR JOINT WORK IN THE PROJECT GUT MICROBIOME OF BOLIVIAN HOWLER MONKEYS
***(Alouatta caraya* and *Alouatta sara*)**

This letter of intent expresses the commitment and interest in collaborative work between the Department of Evolutionary Biology & Ecology of Université libre de Bruxelles(EB&E, ULB) and the Noel Kempff Mercado Natural History Museum (MHNNKM), Biodiversity Research Center from the School of Agricultural Sciences, UAGRM, to carry out the master's thesis study of Maxime Mees under the direction of Professor Jean-François Flot and Dr. Felipe Ennes Silva. The study in Bolivia will have the advice of Dr. Damián I. Rumiz, from the MHHNKM to identify groups of Bolivian howler monkeys and collect fecal samples that allow analyzing the diversity of their microbiome in different ecological conditions. The validity period of this agreement will be 2 years from the date of its signature.

The EB&E Department, Université libre de Bruxelles, is committed to:

- Support and supervise master's candidate Maxime Mees in his analysis of the microbiome of Bolivian manechi monkeys in order to identify differences in the diversity of intestinal microorganisms in each species and under different ecological conditions.
- Coordinate with Ms. Kathia Rivero, responsible for the Vertebrate Zoology area of the MHNNKM, the cataloging tasks and obtaining the export permit for the samples.
- Ensure mention of the MHNNKM, and the participation of the personnel directly involved, in publications or technical documents that use or cite the results of joint work.
- Give a practical theoretical mini-course to MHNNKM staff and local biology students on the scientific foundation, the collection protocol and applications of primate microbiome analysis.

The Noel Kempff Mercado Natural History Museum, as an academic entity and Authorized Scientific Institution (ICA) by the Competent National Authority of the National State, is committed to:

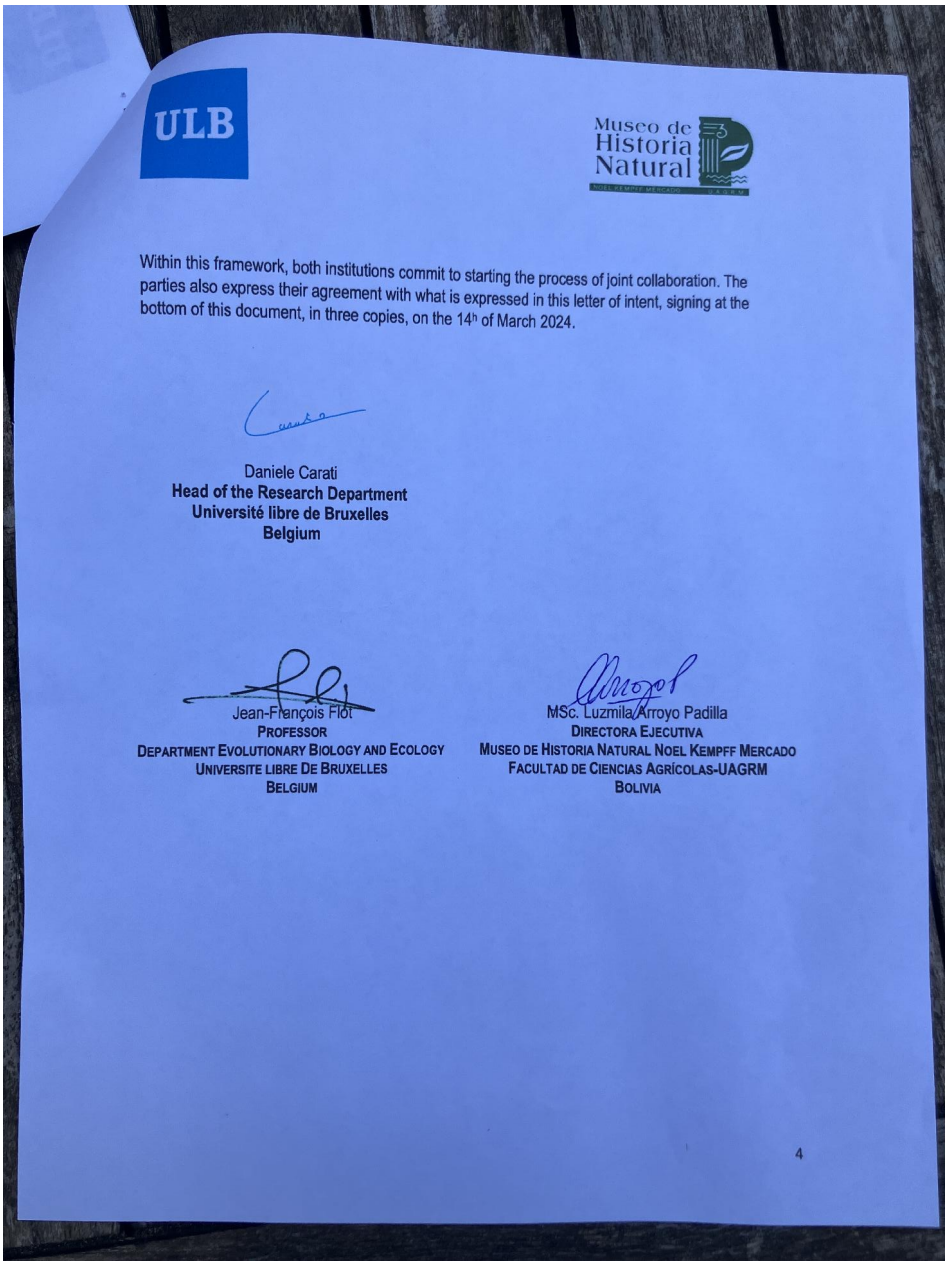
- Participate with technical personnel and advise on the implementation of sampling of the primate microbiome within the framework of the MHNNKM's blanket project "Diversity and conservation of vertebrates in Bolivia: Updating knowledge and research priorities" approved by the General Directorate of Biodiversity and Protected Areas (DGBAP).
- Designate Dr. Damián I. Rumiz as responsible interlocutor in order to ensure a uniform communication channel and better coordination of joint activities.
- Mention the participation of the EB&E Department, Université libre de Bruxelles in publications or technical documents that use or cite the results of joint work.

In case of contradiction, the English version of this agreement shall prevail over the Spanish version.

LAST

3

Figure S4. Letter of intent - English version



ULB

Museo de Historia Natural

Within this framework, both institutions commit to starting the process of joint collaboration. The parties also express their agreement with what is expressed in this letter of intent, signing at the bottom of this document, in three copies, on the 14h of March 2024.

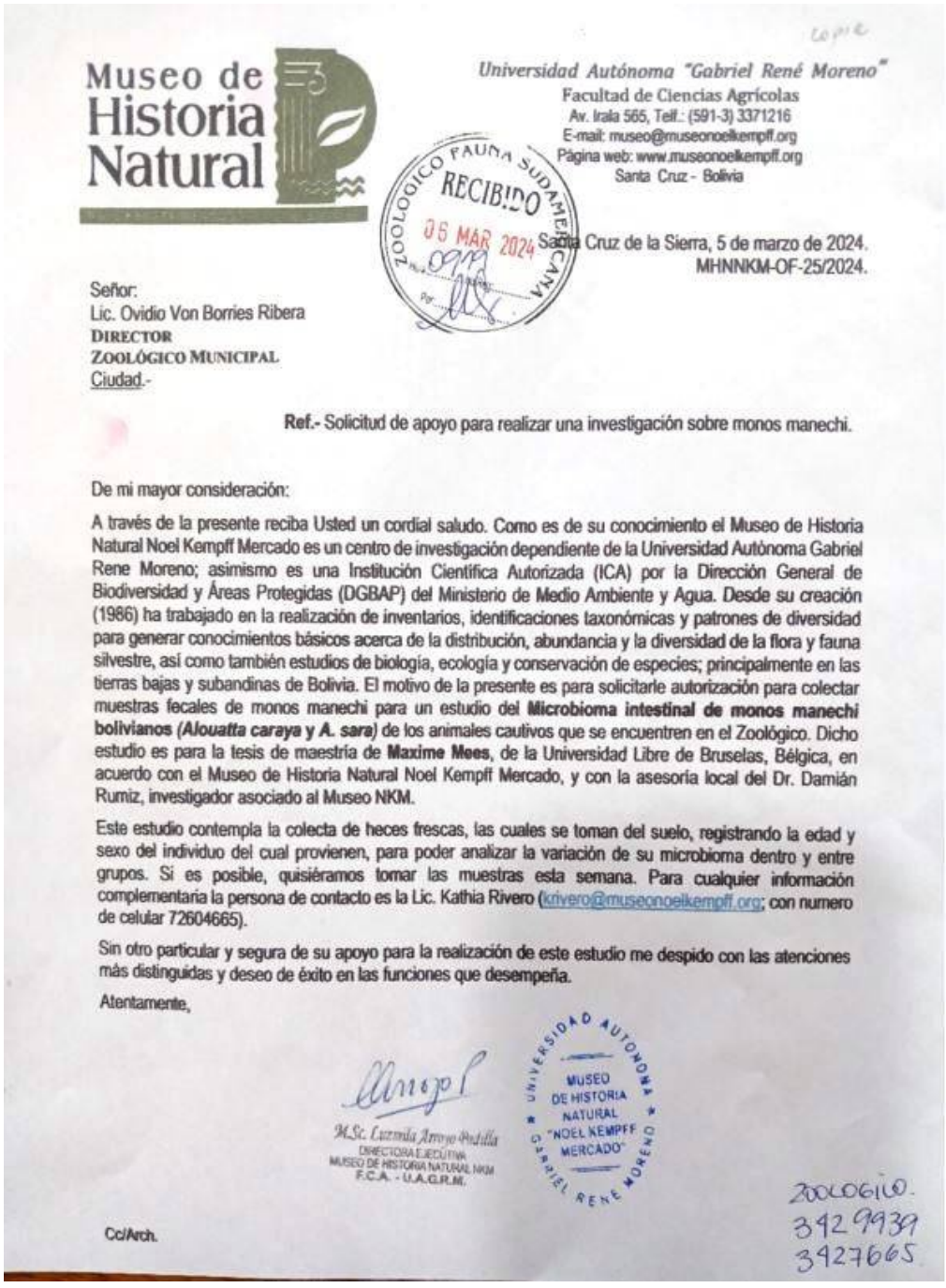
Daniele Carati
Head of the Research Department
Université libre de Bruxelles
Belgium

Jean-François Flot
PROFESSOR
DEPARTMENT EVOLUTIONARY BIOLOGY AND ECOLOGY
UNIVERSITE LIBRE DE BRUXELLES
BELGIUM

MSc. Luzmila Arroyo Padilla
DIRECTORA EJECUTIVA
MUSEO DE HISTORIA NATURAL NOEL KEMPFF MERCADO
FACULTAD DE CIENCIAS AGRÍCOLAS-UAGRM
BOLIVIA

4

Figure S5. Letter of intent - English version



Museo de Historia Natural

Universidad Autónoma "Gabriel René Moreno"
Facultad de Ciencias Agrícolas
Av. Irala 565, Telf.: (591-3) 3371216
E-mail: museo@museonoelkempff.org
Página web: www.museonoelkempff.org
Santa Cruz - Bolivia

ZOOLOGICO FAUNA SUDAMERICANA
RECIBIDO
06 MAR 2024

Santa Cruz de la Sierra, 5 de marzo de 2024.
MHNNKM-OF-25/2024.

Señor:
Lic. Ovidio Von Borries Ribera
DIRECTOR
ZOOLÓGICO MUNICIPAL
Ciudad.-

Ref.- Solicitud de apoyo para realizar una investigación sobre monos manechi.

De mi mayor consideración:

A través de la presente reciba Usted un cordial saludo. Como es de su conocimiento el Museo de Historia Natural Noel Kempff Mercado es un centro de investigación dependiente de la Universidad Autònoma Gabriel Rene Moreno; asimismo es una Institución Científica Autorizada (ICA) por la Dirección General de Biodiversidad y Áreas Protegidas (DGBAP) del Ministerio de Medio Ambiente y Agua. Desde su creación (1986) ha trabajado en la realización de inventarios, identificaciones taxonómicas y patrones de diversidad para generar conocimientos básicos acerca de la distribución, abundancia y la diversidad de la flora y fauna silvestre, así como también estudios de biología, ecología y conservación de especies; principalmente en las tierras bajas y subandinas de Bolivia. El motivo de la presente es para solicitarle autorización para colectar muestras fecales de monos manechi para un estudio del **Microbioma intestinal de monos manechi bolivianos (*Alouatta caraya* y *A. sara*)** de los animales cautivos que se encuentren en el Zoológico. Dicho estudio es para la tesis de maestría de **Maxime Mees**, de la Universidad Libre de Bruselas, Bélgica, en acuerdo con el Museo de Historia Natural Noel Kempff Mercado, y con la asesoría local del Dr. Damián Rumiz, investigador asociado al Museo NKM.

Este estudio contempla la colecta de heces frescas, las cuales se toman del suelo, registrando la edad y sexo del individuo del cual provienen, para poder analizar la variación de su microbioma dentro y entre grupos. Si es posible, quisiéramos tomar las muestras esta semana. Para cualquier información complementaria la persona de contacto es la Lic. Kathia Rivero (krivero@museonoelkempff.org; con numero de celular 72604665).

Sin otro particular y segura de su apoyo para la realización de este estudio me despido con las atenciones más distinguidas y deseo de éxito en las funciones que desempeña.

Atentamente,

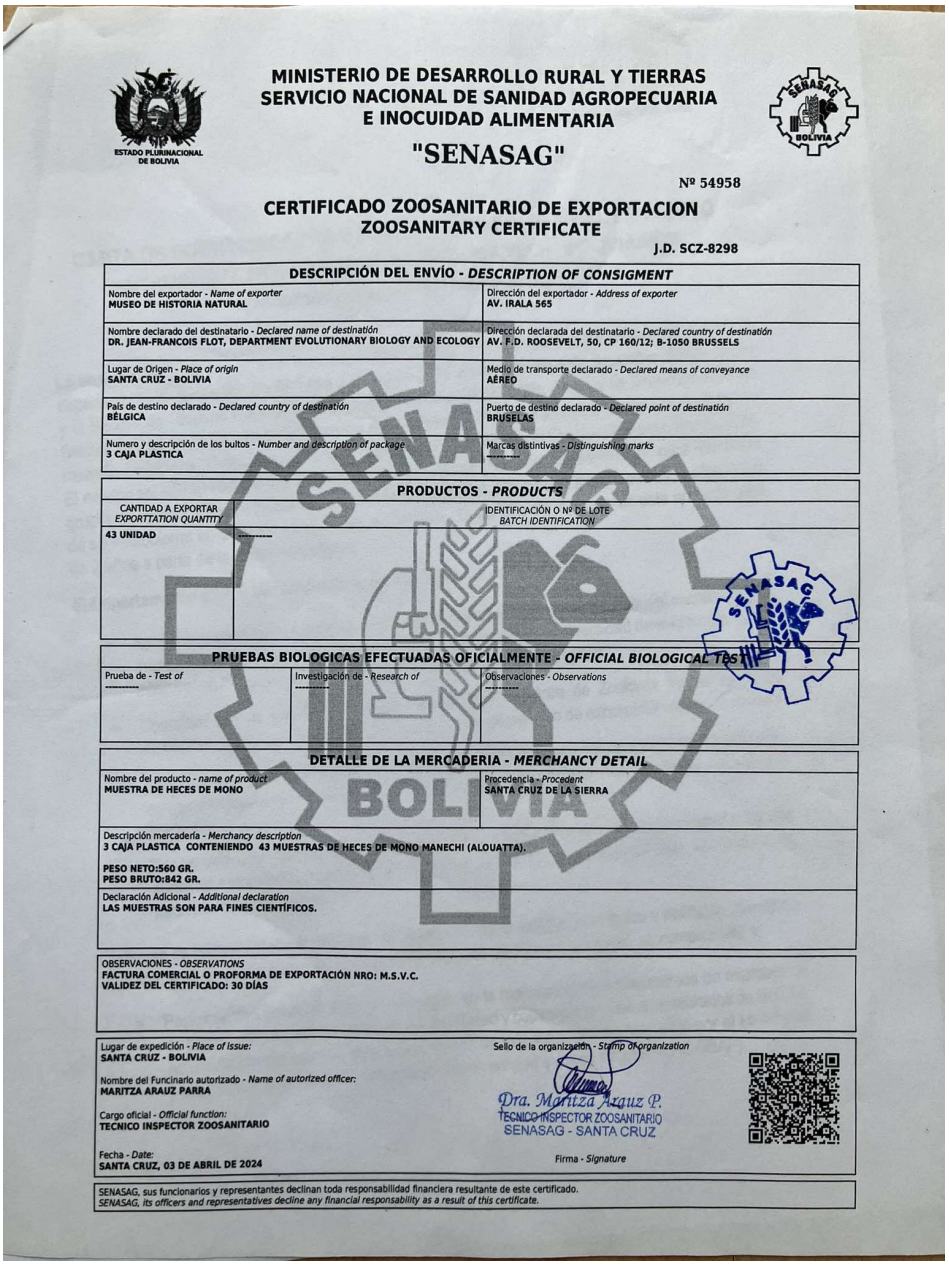
M.Sc. Luzmila Arroyo Padilla
DIRECTORA EJECUTIVA
MUSEO DE HISTORIA NATURAL NKM
F.C.A. - U.A.G.R.M.

UNIVERSIDAD AUTÓNOMA GABRIEL RENÉ MORENO
MUSEO DE HISTORIA NATURAL "NOEL KEMPFF MERCADO"

Cc/Arch.

3429939
3427665

Figure S6. Letter of intent - English version



MINISTERIO DE DESARROLLO RURAL Y TIERRAS
SERVICIO NACIONAL DE SANIDAD AGROPECUARIA
E INOCUIDAD ALIMENTARIA

"SENASAG"

ESTADO PLURINACIONAL DE BOLIVIA

Nº 54958

CERTIFICADO ZOOSANITARIO DE EXPORTACION
ZOOSANITARY CERTIFICATE

J.D. SCZ-8298

DESCRIPCIÓN DEL ENVÍO - *DESCRIPTION OF CONSIGMENT*

Nombre del exportador - *Name of exporter* **MUSEO DE HISTORIA NATURAL**	Dirección del exportador - *Address of exporter* **AV. IRALA 565**
Nombre declarado del destinatario - *Declared name of destinatión* **DR. JEAN-FRANCOIS FLOT, DEPARTMENT EVOLUTIONARY BIOLOGY AND ECOLOGY**	Dirección declarada del destinatario - *Declared country of destinatión* **AV. F.D. ROOSEVELT, 50, CP 160/12; B-1050 BRUSSELS**
Lugar de Origen - *Place of origin* **SANTA CRUZ - BOLIVIA**	Medio de transporte declarado - *Declared means of conveyance* **AÉREO**
País de destino declarado - *Declared country of destinatión* **BELGICA**	Puerto de destino declarado - *Declared point of destinatión* **BRUSELAS**
Numero y descripción de los bultos - *Number and description of package* **3 CAJA PLASTICA**	Marcas distintivas - *Distinguishing marks* ----------

PRODUCTOS - *PRODUCTS*

CANTIDAD A EXPORTAR *EXPORTTATION QUANTITY*	IDENTIFICACIÓN O Nº DE LOTE *BATCH IDENTIFICATION*
43 UNIDAD	----------

PRUEBAS BIOLOGICAS EFECTUADAS OFICIALMENTE - *OFFICIAL BIOLOGICAL TEST*

Prueba de - *Test of*	Investigación de - *Research of*	Observaciones - *Observations*
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DETALLE DE LA MERCADERIA - *MERCHANCY DETAIL*

Nombre del producto - *name of product* **MUESTRA DE HECES DE MONO**	Procedencia - *Procedent* **SANTA CRUZ DE LA SIERRA**

Descripción mercadería - *Merchancy description*
3 CAJA PLASTICA CONTENIENDO 43 MUESTRAS DE HECES DE MONO MANECHI (ALOUATTA).

PESO NETO:560 GR.
PESO BRUTO:842 GR.

Declaración Adicional - *Additional declaration*
LAS MUESTRAS SON PARA FINES CIENTIFICOS.

OBSERVACIONES - *OBSERVATIONS*
FACTURA COMERCIAL O PROFORMA DE EXPORTACIÓN NRO: M.S.V.C.
VALIDEZ DEL CERTIFICADO: 30 DÍAS

Lugar de expedición - *Place of issue:*
SANTA CRUZ - BOLIVIA

Nombre del Funcinario autorizado - *Name of autorized officer:*
MARITZA ARAUZ PARRA

Cargo oficial - *Official function:*
TECNICO INSPECTOR ZOOSANITARIO

Fecha - *Date:*
SANTA CRUZ, 03 DE ABRIL DE 2024

Sello de la organización - *Stamp of organization*

Dra. Maritza Arauz P.
TECNICO INSPECTOR ZOOSANITARIO
SENASAG - SANTA CRUZ

Firma - *Signature*

SENASAG, sus funcionarios y representantes declinan toda responsabilidad financiera resultante de este certificado.
SENASAG, its officers and representatives decline any financial responsability as a result of this certificate.

Figure S7. Zoosanitary certificate

Annex 2: Results of the errors encountered during the development of the methodology

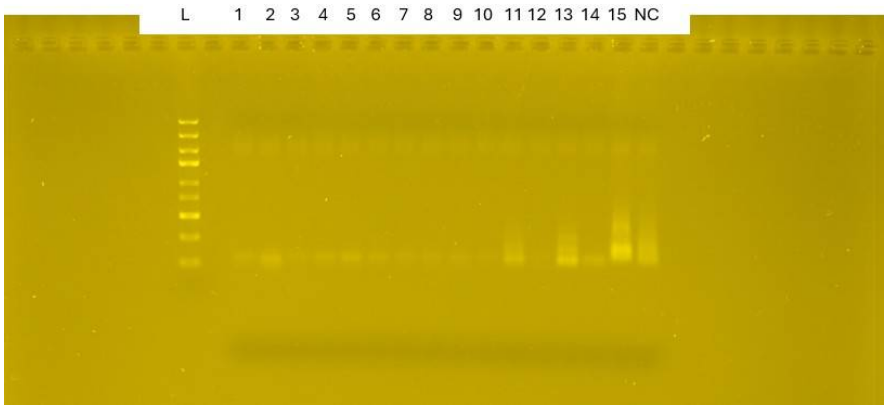

Figure S8. Electrophoresis on an agarose gel performed following PCR amplification of samples 1 to 15 ; L = Ladder (Thermo Scientific O'GeneRuler Express DNA Ladder, ready-to-use) ; NC = negative control

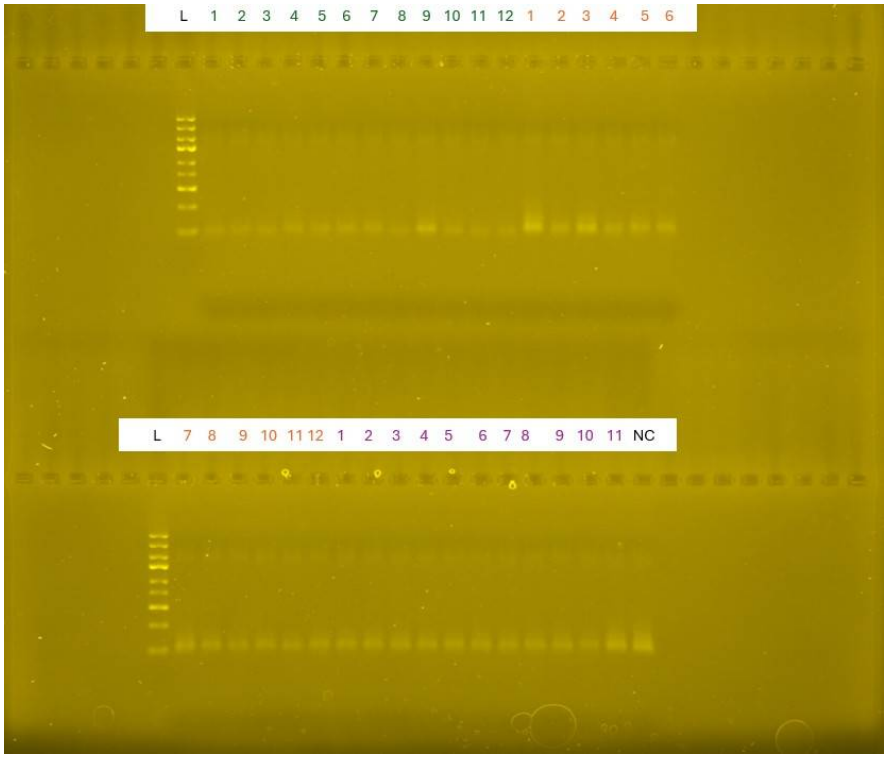

Figure S9. Electrophoresis on an agarose gel performed following PCR amplification of samples 1 to 3 using a temperature gradient

Table S1. Results of the electrophoresis performed on agarose gel after PCR amplification of samples 11, 12, and 13 using a temperature gradient

N°	Sample N°	Temperature (°C)	Result
1	11	42.9	X
2	11	43.2	X
3	11	43.9	X
4	11	45.1	X
5	11	46.7	X
6	11	48.3	X
7	11	49.7	X
8	11	51.3	X
9	11	52.9	X
10	11	54.1	X
11	11	54.8	X
12	11	55.2	X
1	12	42.9	X
2	12	43.2	X
3	12	43.9	X
4	12	45.1	X
5	12	46.7	X
6	12	48.3	X
7	12	49.7	X
8	12	51.3	X
9	12	52.9	X
10	12	54.1	X
11	12	54.8	X
12	12	55.2	X
1	13	42.9	X
2	13	43.2	X
3	13	43.9	X
4	13	45.1	X
5	13	46.7	X
6	13	48.3	X
7	13	49.7	X
8	13	51.3	X
9	13	52.9	X
10	13	54.1	X
11	13	54.8	X

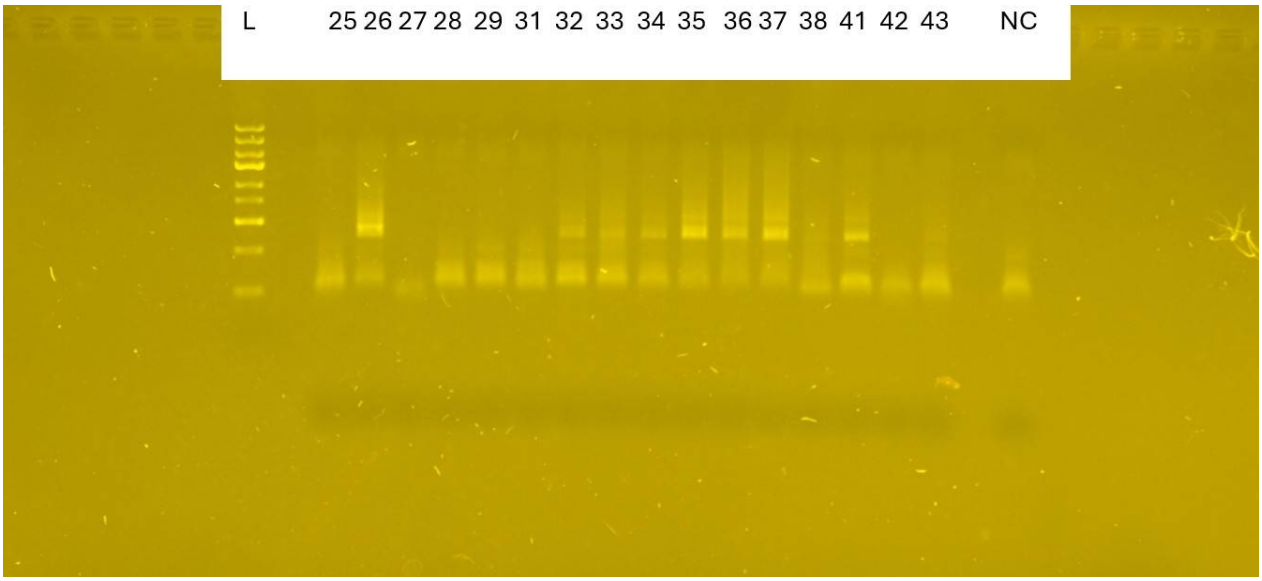

Figure S10. Electrophoresis on an agarose gel performed following PCR amplification of samples 25 to 43 using cytochrome b primers

Annex 3: Alpha and beta diversity plots of the effect of habitat on Alouatta caraya

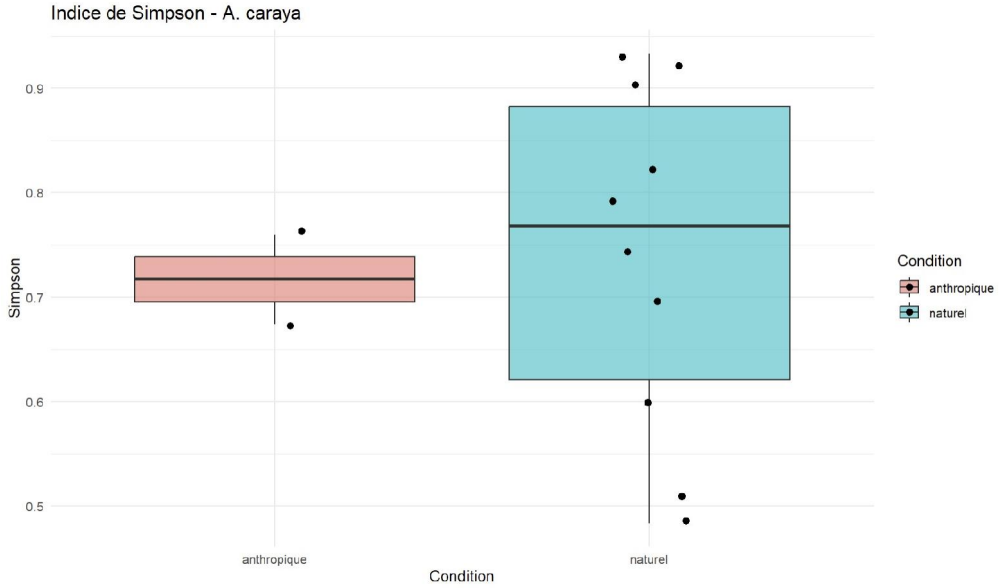

Figure S11. Simpson index in Alouatta caraya by habitat type

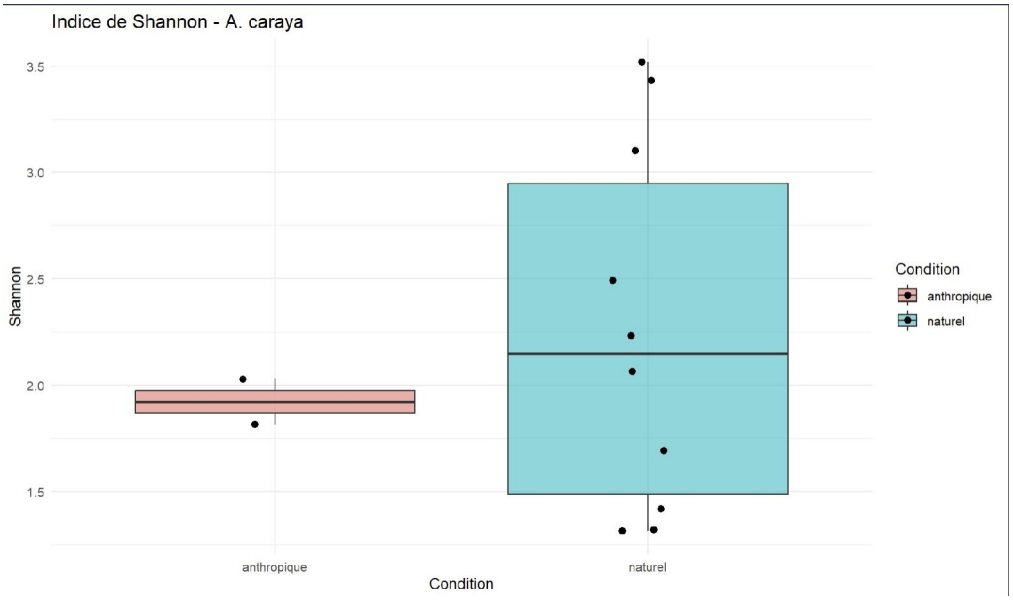

Figure S12. Shannon index in Alouatta caraya by habitat type

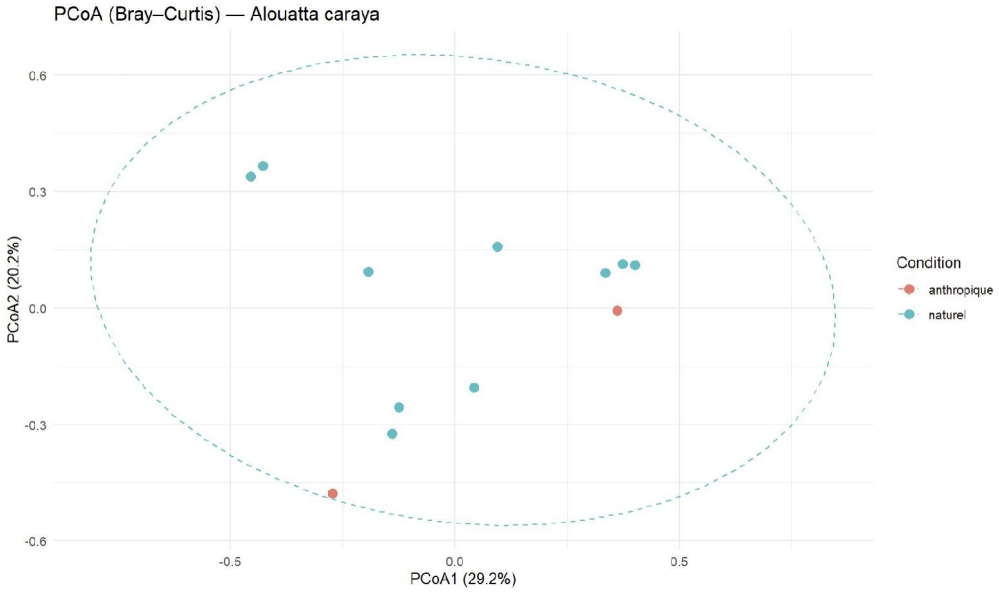

Figure S13. PCoA of the effect of habitat on microbiome composition in Alouatta caraya

Annex 4: Comparaison of DNA extraction kit

The fecal samples are lysed through both chemical and mechanical homogenization. The recovered lysate is then purified to remove inhibitors. Following the cleanup, the lysate is passed through a silica spin filter filtration membrane. The membrane is subsequently washed twice before eluting the DNA bound to the silica. This process yields highly purified DNA, ensuring a 100% success rate for PCR amplification during testing.

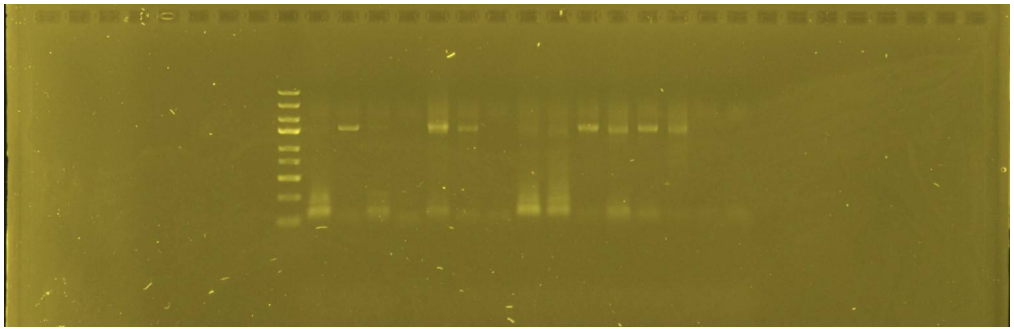

Figure S14. PCR on the 13 DNA extractions from my supervisor obtained with the DNA extraction kit from Sylphium®

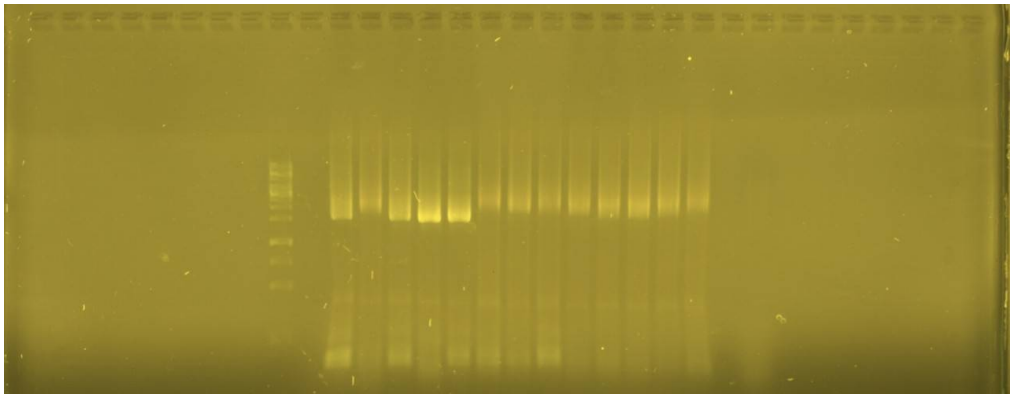

Figure S15. PCR on the 13 DNA extractions from my supervisor obtained with the DNA extraction kit from QIAGEN®