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Escuela de Ciencias Biológicas e Ingeniería

Analysis of Oral Microbiota in Bats

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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Dedicatoria

A mis padres, Gloria y Luis, quienes me impulsan a seguir adelante día a día. Gracias por cada palabra de aliento, por su inmenso esfuerzo y dedicación, por su ilimitada paciencia y su amor incondicional. Gracias por prepararme para los altibajos de la vida. Ustedes son mi modelo a seguir.

A mis hermanos, Majo y Joshua, por estar siempre a mi lado dándome apoyo.

Keila Franchesca Gómez Acosta

Resumen

Los murciélagos son considerados los únicos mamíferos voladores que cumplen varias funciones ecológicas y económicas esenciales para animales, plantas y humanos. Sin embargo, la actividad humana y los cambios climáticos que han ocurrido en los últimos años están afectando los ecosistemas y sus hábitos alimenticios, disminuyendo así la población de diferentes especies de murciélagos. Por otro lado, los murciélagos son reservorios de diversos patógenos que afectan la salud de humanos y animales. Por esta razón, esta investigación se enfoca en analizar la microbiota oral de los murciélagos para encontrar una relación entre la salud y las enfermedades presentes en estos mamíferos. Además, en comparación con otros hábitats corporales como las heces o la orina, muestran similitudes y diferencias entre las poblaciones bacterianas. El estudio se realizó en tres áreas diferentes dentro del cantón Urcuquí ubicado en la provincia de Imbabura. Muestras de saliva fueron recolectadas de especies de la familia Phyllostomidae. Finalmente, estudios comparativos fueron diseñados usando saliva para identificar bacterias utilizando métodos de cultivo y herramientas metagenómicas. Se enfrentaron varios problemas técnicos en el estudio. Por lo tanto, se sugiere continuar con este estudio para concluirlo y también para comparar otros microhábitats del cuerpo con diferentes especies animales para encontrar especificidad en las bacterias que albergan estos sitios. Además, se pueden diseñar algunas técnicas para evitar que los murciélagos transmitan enfermedades que afectan a humanos y animales sin alterar la población de estos mamíferos voladores.

Palabras clave:

Murciélagos, Quirópteros, Microbioma oral, Enfermedades infecciosas emergentes, Cavidad bucal, Población microbiana, Comunidades microbianas, Cavidad oral, Microflora oral, Ecología de murciélagos, Saliva.

Abstract

Bats are the only volant mammals. By occupying a diverse range of ecological niches, they fulfill several essential ecological and economic functions for other groups of animals, plants, and humans alike. However, human activity and the recent global climatic changes are affecting their ecosystems and the eating populations of bat species. On the other hand, bats are reservoirs for various pathogens that affect humans and animals health. For this reason, this investigation focuses on analyzing the oral microbiota of bats to find a relationship between health and the diseases present in these mammals. Besides, when compared to other bodily habitats such as feces or urine, they show similarities and differences between bacterial populations. The study was conducted at three different locations within the canton Urcuquí located in the province of Imbabura. Saliva samples were collected from species of the Phyllostomidae. Finally, comparated my findings with those of studies using saliva to identify bacteria through culture methods and metagenomic tools. A great diversity bacteria was found. Some of them likely are part of healthy oral microbiota of bats. Therefore, I suggested to continue with this type of study to compare other microhabitats of body within different animal species to determine the specificity of bacteria to different bodily cavities. The findings of this type of study could eventually lead to the development of techniques that could prevent bats from transmitting diseases that affect humans and animals, all without altering the population of these flying mammals.

Key words:

Bats, Chiroptera, Oral microbiome, EIDs, Buccal cavity, Microbial population, Microbial communities, Oral cavity, Oral microflora, Bat ecology, Saliva.

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Abbreviations

EIDs: Emerging Infectious Diseases

S.Y: Sculptures Yachay Tech

Y.B.G: Yachay Botanical Garden

TE buffer: Tris – EDTA buffer

CTAB: Cethyl trimethyl ammonium bromide

PVP: Polyvinylpyrrolidone

EDTA: Ethylenediaminetetra acetic acid

OTUs: Operational taxonomic units

1. Introduction

Among the mammal orders that are most widely distributed around the world are the bats (order Chiroptera). Bats are the most diverse mammalian gruop, and as such they exhibit many distinctive ecological and physiological adaptations (Kasso & Balakrishnan, 2013; Mühldorfer, 2013). Ecuador contains more than 100 species of bats in the rainforest region alone, or 12% of the world's known bat species (*Mammals of Ecuador*, 2017). The number increases to 173 species of bats for the entire country (BCI, n.d.). Of these, 19 are threatened, representing 17% of the total biodiversity of mammals in Ecuador (Burneo & Tirira, 2014). In general, little is known about the biodiversity, behavior, ecological and economic importance of bats in Ecuador.

Bats contribute to the balance of ecosystems by controlling insect populations (Calisher et al., 2006a). Moreover, bats belong to many feeding guilds, being able to feed on insects, mammals, fish, blood fruits, or pollen. Most bat species use echolocation to move and to find prey. Additionally, bats are found on all continents except Antarctica. It is known that bats are reservoirs of viruses and bacteria that can infect humans and other animals, and this is one of the reasons why they are being eradicated causing their populations to decline with critical consequences to ecosystems (Calisher et al., 2006b).

Furthermore, bats have great potential as bioindicators (environmental and ecological indicators) because trends in their populations can be determined; and short and long- term effects on communities can be measured. In addition, they reflect the condition of plant populations on which they feed and pollinate. Because bats occupy such a wide array of ecological niches, they offer a critical multisensory proxy for assessing ecosystem health (Jones et al., 2009). Furthermore, some factors are affecting bat populations, such as the alteration of ecosystems due to climate change, human activities including agriculture, industries or urbanization, or diseases. For example, in Ecuador, the areas where bats live are threatened by deforestation and fragmentation (Burneo & Tirira, 2014). Thus, it is essential to monitor and control the population of bats.

There is a wide variety of emerging infectious diseases, which create a risk to global biodiversity and humans alike. The increase in human activities, in turn,

exposes bats, which increases the transmission of diseases between bats and humans or other animals especially domestic animals or livestock; (Hayman et al., 2013). For instance, rabies is one of the infectious diseases with a very high mortality rate and is one of the most studied associated with Chiroptera. Other viruses that bats can present are filoviruses, henipaviruses, lyssavirus, paramyxoviruses, and coronaviruses (Kuzmin et al., 2011), which can cause health problems for humans such as hemorrhages, respiratory issues, and even death. More research focuses on zoonotic viral infections in bats rather than bacterial and fungal infections. These latter two types of infections may have similar ecological generalities, therefore, it is substantial to understand the ecology of bats to know these pathogens influence on overall disease dynamics (Hayman et al., 2013).

There is a well-known association between bats and pathogenic fungi. Different types of fungi have been isolated in bat species, for example, *Histoplasma capsulatum, Paracoccidiodes brasiliensis, Sporothrix schenckii, Trichophyton mentagrophytes, Microsporum gypseum*, and *M. canis*. Some fungi like *Geomyces destructans*, which is the causative agent of White Nose Syndrome (WNS), and it has killed over 5.7 million bats in North America, are highly pathogenic. Wing lesions and mycelial growth on the bat´s muzzle and body characterize the WNS (Johnson et al., 2013). This disease is considered a severe threat to mammalian diversity as there are about 1200 bat species in all, representing approximately 20% of all known mammal species (Voyron et al., 2010). Other fungi such as *Cladosporium, Fusarium, Mortierella*, and *Penicillium* are often isolated on the wings of bats that can be found in caves or mines (Johnson et al., 2013).

Conversely, an area that requires further investigation is the microbiome of bats. This microbial ecosystem can be considered part of the healthy biota of these mammals, particularly digestive tract (Galicia et al., 2014). At the same time, changes in the microbiome can give way to a series of systemic diseases, including oral diseases (Gao et al., 2018). Some bacteria found in this microhabitat are causative agents of diseases that can affect humans and other animals. On the other hand, they could also be part of the bats healthy microbiota, as is the case of *E. coli* (Galicia et al., 2014). There is a great need for more

studies on the oral microbiome in bats; their symbiotic relationship with their host and the other microbes (Galicia et al., 2014), population dynamics (Dietrich et al., 2018), species composition (Dietrich et al., 2017), and organization and functional relationships (Gao et al., 2018) of oral microbiome and the bacterial community that are housed in the oral cavity of the chiropterans could prevent diseases that can affect the bat populations, other animals and humans.

The objective of this research was to characterize the oral microbiota of bats as a preliminary step to ascertain what types of infectious diseases could affect the population of bats. The collection of saliva samples will be used to identify the specific characteristics of bats as carriers of pathogens to know if there is a relationship with their state of health and diseases associated with the oral cavity. Furthermore, this study will show the different ecological roles and their importance, the diversity of the bacterial population in the microbiota of bats, and its relationship with infectious emerging diseases.

In this study will also review current studies in the field of the oral microbiome of bats, including the methods they use to characterize bacterial populations. The limited available information on this subject can be explained by the difficulties in collecting and preserving saliva samples, there are more studies focused on the more easily sampled gastrointestinal bacterial flora and bacterial characterization through genetic techniques of blood and ectoparasites. (Mühldorfer, 2013). For this reason, the most current scientific findings will be reviewed, with the aim of stimulating research related to this topic. In summary, the oral cavity is a diverse and structured ecosystem in which the interactions among its microorganisms related to each ones habitat. In addition, understanding how bats coexist with viruses, pathogens, bacteria, or other microorganisms without showing any symptoms is essential for the development of therapies that can help other animals such as humans.

1.1 Important Roles of Bats Ecology

Bats are one of the most important groups in the animal kingdom and one of the most numerous and diverse within mammals (Ducummon, 2000). They are bioindicators that are indispensable for ecosystems because they play different

ecological roles such as, seed dispersers, prey and predators, parasite hosts, pollinator, nutrient cyclers, and contributing to soil fertility (Kasso & Balakrishnan, 2013). In addition, bats are economically important for several reasons. They are biological pest controllers, their guano is used as a fertilizer in agricultural fields; and they are pollinators of different plant species. Bats are also employed as medicine, their seed dispersal mechanism maintains tropical forests and they are essential for tourism and research, among other reasons (Kasso & Balakrishnan, 2013).

In addition, bats are a fundamental element of mammalian biodiversity in the Neotropical zone. However, bat populations are decreasing due to the fragmentation and destruction of tropical forests (García-García & Santos-Moreno, 2014) and other environmental factors (Jones et al., 2009). In recent years, our planet has undergone severe climate alterations, and owing to the increase of the human population; there is a large-scale loss of habitats worldwide (Jones et al., 2009; Parmesan, 2006). Ecosystems respond to anthropogenic changes and, as a result, habitat conversion and climate change have widespread effects on diversity that are reflected by, indicators that have different applications (McGeogh, 1998). Within the classification of bioindicators, there are three types; environmental, biodiversity, and ecological (Jones et al 2009). Bats can be crucial ecological and environmental indicators because they can respond to a wide range of environmental stressors, as shown in Figure 1.

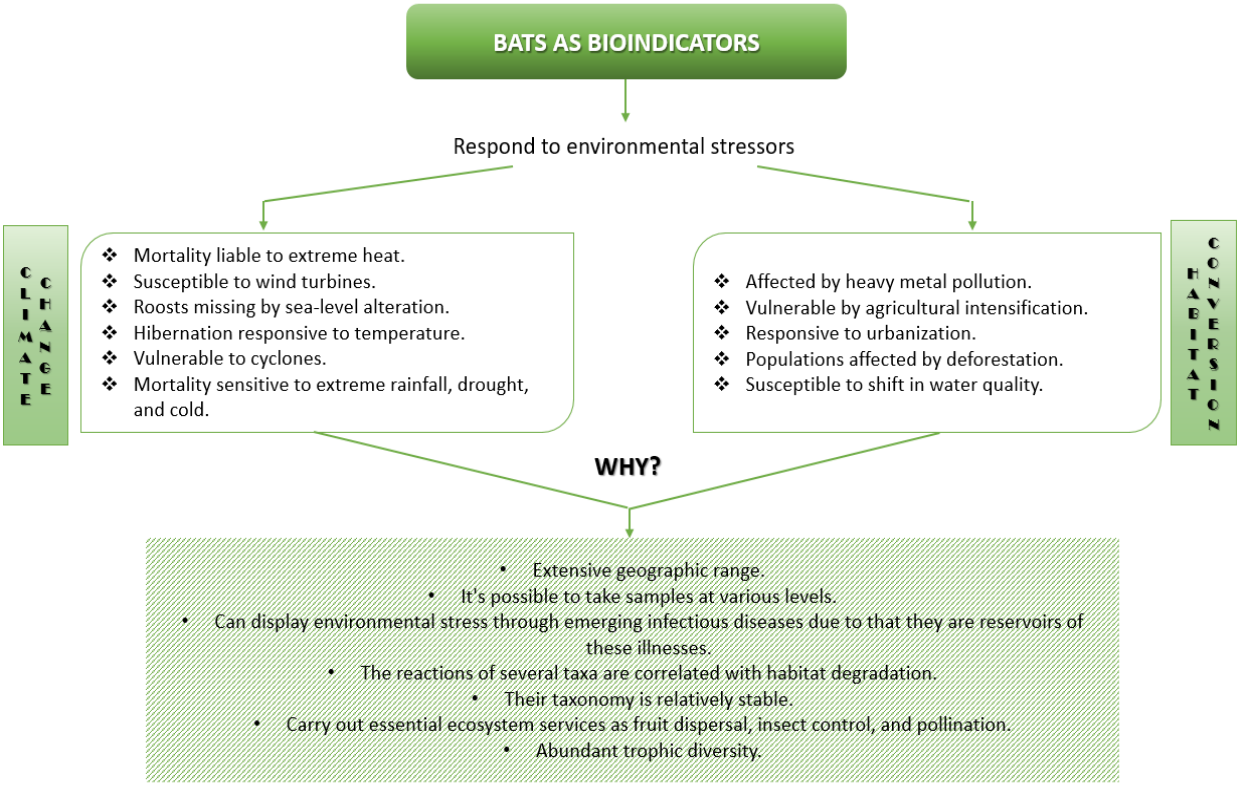

Figure 1: Bats as bioindicators (Jones et al., 2009).

The relationship between trophic levels, pollution, and environmental disturbance allows bats and especially insectivores to be excellent bioindicators. They tend to show the consequences of those alterations before other organisms such as insects or birds (Fenton et al., 1992) owing to their longevity, size, distribution, and mobility (Kasso & Balakrishnan, 2013; Russo & Jones, 2015). We need to control and monitor these mammals to not only know about their state but also to know the condition of their ecosystems and take precautionary measures.

Bats are considered social animals living in small to large groups (Kunz & Fenton, 2005; Kuzmin et al., 2011). While they feed, during mother-child interactions courtship and mating or any other social interactions, bats use the senses of smell and sight, or they use tactile, acoustic or thermal signals to communicate. For example, the males emit perceptible vocalizations, leave odor marks in the mating territories and their females, and finally show their flight skills. All these characteristics are part of the evolution of the bat mating system, which provides stability in social groups (Kunz & Fenton, 2005).

Bats are capable of flight enabling them to be a very diverse group to have a worldwide distribution. They are the only mammals that have this ability, which allows them to rapidly disperse emerging infectious diseases (EIDs) (Jones et al., 2009; Kuzmin et al., 2011). Indeed, the flying skills that confer them the anatomical characteristics of bats are related to immunological features (Kuzmin et al., 2011). Flight has not only influenced their eating habits and reproductive strategies but also has been implicated in their roosting behavior (Jones et al., 2009). For example, roosts-sites are variable, and these can be; caves, mines, tree hollows, rock crevices, human-made structures (Brigham et al., 1997; Callahan et al., 1997; Ducummon, 2000; Jones et al., 2009; Kuzmin et al., 2011), buttress cavities, branches, tree trunks, exposed boles, bird nests, bamboo culm, holes beneath exfoliating bark (Jones et al., 2009; Kunz & Fenton, 2005; Vonhof & Barclay, 1996). Clearly, bats have a wide variety of ecological niches, and choice of roosting places depends on different factors. However, with increasing deforestation look for agricultural areas or man-made structures bats increasingly look for human made structures (Kuzmin et al., 2011). Some use the mines to protect against the cold or winter and for hibernation (Ducummon, 2000).

There are few animals specialized in feeding on bats (Lima & O'Keefe, 2013). Among the predators of bats are birds, amphibians, reptiles, fish, and other mammals (Kasso & Balakrishnan, 2013; Lima & O'Keefe, 2013). Some studies show that bats are easy prey during the day rather than night (Rydell & Speakman, 1995). Owls, hawks, falcons attack Chiroptera in caves. Raccoons, cats, and snakes usually attack bats in tree trunks, caves, or sites where there is a great concentration of bats. Bats are auditory animals (Fenton et al., 1994; Lima & O'Keefe, 2013) and they use acoustic signals to detect predators or send distress calls to other bats. Echolocation, vision, and chemical signals are little studied topics about dangerous situations for bats (Lima & O'Keefe, 2013).

Bats as predators have multiple feeding strategies. Some eat spiders, scorpions, mosquitoes, midge, moths, and beetles. Others are predators of small mammals, frogs, fish, and blood of birds and mammals (Kasso & Balakrishnan, 2013). Some use vision for feeding (sanguinivorous, frugivorous, nectarivorous). Another stimulus that they use to capture prey is sound. They use echolocation, prey-generated sound to track, detect, and evaluate potential victims. The vampire bats use thermal detection to detect prey (Kunz & Fenton, 2005). The

predation of bats generates direct and indirect effects. Direct effects can be observed in herbivore communities whereas indirect effects are reflected by plant communities (Kasso & Balakrishnan, 2013).

The study of the bats feeding ecology can be studied through feces and stomach contents. In this way, species of animals or seeds which they consume in their diet can be known (M. Brock Fenton, 1982). Feeding behavior varies according to age, sex, colony size, reproductive status, seasonal changes, availability of food resources (Kunz, 1974), and trophic specialization (Kuzmin et al., 2011). Frugivorous bats feed on the ripe fruits of various species. The main trees species are *Ficus, Pipers, Solanum*, and *Crecopia* sp. (González, 1998; Lou & Yurrita, 2005). Bats often leave partial fruit remains, which can become infected with viral particles that can then be consumed by other animals (Kuzmin et al., 2011). Fruit consumption is indispensable for seed dispersal (Kasso & Balakrishnan, 2013; Kuzmin et al., 2011). In contrast, omnivorous bats consume plants, nectar, small vertebrates, and arthropods (Kuzmin et al., 2011).

Another ecological service is pollination, which allows the reproductive success of some plants (Kuzmin et al., 2011). The Phyllostomidae pollinate approximately 360 species of plants. Their morphology is designed to consume pollen and nectar (Fleming et al., 2009). For this reason, some bats have an elongated tongue and snout (Kuzmin et al., 2011). The characteristics of the bat-pollinated flowers have white or green coloration, cauliflory, nocturnal anthesis (less colorful), musty or fetid smell, radially or tubular flowers, shaving brush shape and they produce hexose-rich nectar. (Fleming et al., 2009). In economic terms, pollination benefits the cultivation of fruits such as breadfruits, bananas, agave, mangos, petai, and durians.

The dispersion of seeds through bats, in turn, allows several species of vertebrates to feed on the fruits produced by those plants. This ecological role is critical because it influences forest regeneration (Kasso & Balakrishnan, 2013). Therefore, pollination and seed dispersal are a pivotal to both economic and ecological systems because they prevent the loss of many species of animals and plants.

Furthermore, bats contribute to biological pest control because they consume large numbers of insects during the night (Kasso & Balakrishnan, 2013). Bats feed on pests which affect the yield of cash crops such as rice (Wanger et al., 2014), cotton (Federico et al., 2008), corn (Cleveland et al., 2006), cocoa (Maas et al., 2013), coffee (Karp & Daily, 2014), tobacco, sugar beet, tomato, cabbage, grapes (Riccucci & Lanza, 2014), etc. To sum up, insectivorous bats decrease the abundance of nocturnal insects, because they are their main predators. These pests affect different crops giving a tremendous economic impact on society. Thus, bats are biological control agents of these pests (Aguiar & Antonini, 2008), and they are used as an excellent alternative to pesticides (Puig-Montserrat et al., 2015) and other substances that are toxic and harmful to humans and the environment.

In addition, another benefit that bats create is nutrient delivery and soil fertilization. Thanks to these mammals having the ability to fly, they are able to transfer nutrients to the soils of different ecosystems (Kasso & Balakrishnan, 2013). The diversity in their diet and the guano deposit of bats, increase the fertility of the soil (Shetty et al., 2013). Guano contains high nutritional contents (Voigt et al., 2015), necessary for the growth and development of plants. To conclude, bats are highly beneficial not only to the economy of the countries but also entire ecosystems. Consequently, it is essential to know the ecology of bats to come up with plans for their conservation.

1.2 Microbial Community in Bats and EIDs

Bats are involved in the epidemiology of several zoonotic diseases (Galicia et al., 2014). They harbor various microorganisms that include parasites, viruses, fungi, and bacteria (Mühldorfer, 2013). The coevolution between viruses and bats could explain the role of this mammal in EIDs (Kuzmin et al., 2011). Bats are reservoirs (Dietrich et al., 2017; Dobson, 2005) of many diseases because they have immunological characteristics that allow them to survive pathogens and transmit certain viruses (Kuzmin et al., 2011) and bacteria (Calisher et al., 2006a). The most studied pathogens include; lyssavirus, coronavirus, henipavirus, and filovirus (Baker et al., 2013; Kuzmin et al., 2011). Other pathogens of the bat microbiome include *Bartonella* spp. and *Leptospira* spp (Ingala et al., 2018).

First, the ecological diversity of bats determines their eating habits (Dobson, 2005) and, variety roosting places (Kunz & Fenton, 2005) that promote the biodiversity of pathogens (Kuzmin et al., 2011; O'Shea et al., 2014) which in turn affect human and animals health. Thus, diet is one of the main drivers of structure, composition, and function of the microbiome (Ingala et al., 2018). In addition, a physiological characteristic of bats that may be of importance for the microbiome is that these mammals are long-lived. However, it is not known what mechanism microbes use to increase the life span of Chiroptera (Ingala et al., 2018).

Owing to they are unique, flying ability , bats can cover large and diverse geographic areas (Jones et al., 2009). Hence, they facilitate the transmission of EIDs during their outings. Further, this ability gives them certain immunological characteristics (Kuzmin et al., 2011) that could favor viruses. A low body mass is a requirement to fly. Therefore, bats have hollow bones, with very little bone marrow. Thus, they generate B cells in different organs (Kuzmin et al., 2011). During the flight, metabolism and body temperature greatly increase in bats. The underlying metabolic processes could help the immune system by offering selective force to viruses (O'Shea et al., 2014).

On the other hand, it is necessary to learn what type of bacteria are part of the normal microbiota of bats as opposed to causative agents of diseases that can affect humans and animals (Galicia et al., 2014). Communities of bacteria in the intestine, colon, feces, urine, and guano of bats have been studied. However, there is limited research on the bacterial composition of other parts of the body of these mammals (Dietrich et al., 2017). Chiroptera can be used as models to study microbial evolution. Hence, the studies of bats microbiomes could help us understand host-microbe evolution and the ecology and the impact these may have on human and bats health (Ingala et al., 2018).

Those bats that reside in rural and urban ecosystems could contract microorganisms from insects and environmental sources. This could mean that bats are reservoirs of antibiotic-resistant bacteria. However, information is still limited (Mühldorfer, 2013). In addition, some studies show that bat species collected within the same geographic area and ecological niche share ectoparasites such as *Bartonella* (Ingala et al., 2018) thay may transmit a variety of diseases. Nevertheless, bats infected with these pathogens often show no

signs of disease because they are able to control viral replication through innate antiviral mechanisms (Baker et al., 2013).

In conclusion, Intrinsic and extrinsic factors (Mühldorfer, 2013), can expose them to microbial pathogens (Jones et al., 2009; Mühldorfer, 2013). Therefore, analyzing their entire microbiome could help discover bacteria that perform vital functions in host bodily ecosystems (Ingala et al., 2018). For instance, microbiomes contribute to health, digestion, inhibition of pathogens, etc. (Sherrill-Mix et al., 2018). Thus, genetic analysis, fluorescent imaging technology, and sequencing can be used to map the microorganism help us understand the composition and organization of microbial communities that are a dynamic and complex (Welch et al., 2019). Finally, more studies about the microbial composition should be performed in association with the phylogeny, diet, ecology, etc. of the host.

1.3 Oral Microbiota

One of the oldest microbial host bodily ecosystems is the oral microbiota. It is essential to understand how this type of microbiota is composed because it could be a gateway for infections (Coll et al., 2015). The oral cavity has different habitats such as teeth, tongue, cheek surfaces, buccal mucosa, palate, saliva, etc. It creating the appropriate conditions and providing nutrients necessary for the colonization of different microorganisms (Gao et al., 2018; Welch et al., 2019). The microbiota of the oral cavity is diverse and contains about 700 species of bacteria (Belstrøm et al., 2017). These bacteria have specific functions; for example, some support oral homeostasis (Brown, 2019). However, nutritional changes. Also, changes in the immune system produce functional and structural alterations in the oral microbiome (Belstrøm et al., 2017).

The biological fluid that maintains oral health is saliva (Belstrøm et al., 2017). Salivary glands produce certain secretions containing proteins and mucins (Welch et al., 2019) which limit the accumulation of and eliminate of bacteria found on the surface of the teeth (Curtis et al., 2011). Eating habits influence oral microflora with what we might think that microorganisms around the planet could appear in the mouths of animals and humans. These microbes influence spatial distribution

of pH, amount of oxygen, and nutritional components as well as microbial biofilms (Welch et al., 2019).

The characterization of oral microbiomes is limited due to the complexity and high costs of the methods used. Further, cultures and strain tests still need more research to be improved because their knowledge are limited (Sturgeon et al., 2014). Metagenomic and metatranscriptomic approches (Belstrøm et al., 2017) have a better performance when performing analysing of microbial communities in saliva (Belstrøm et al., 2017; Welch et al., 2019). Hence, more research is needed to create new efficient methods that allow the characterizing of uncultivable, cultivable, and unknown bacteria in this microhabitat (Sturgeon et al., 2014).

2. Methods and Materials

2.1 Study area

Bats were captured in two places within the Canton San Miguel de Urcuquí located in Imbabura province. Urcuquí is limited by the Canton Ibarra to the north; Antonio Ante and Cotacachi to the south; and the province of Esmeraldas to the west. The average altitude is 2,384 m.s.a.l. The geographic coordinates 0° 26' 20" N and 78° 11' 50" W (Canton Urcuquí). Its average temperature ranges from 14 °C to 19 °C (Rivera, 2013).

The climate varies from cold, temperate to subtropical, with a humidity of approximately 80% (de la Torre, 2014). The rainy season is peak presented in April and November (Cruz & Armas, 2014). Its average rainfall is for the low zones of and 1,750 mm for its high zones. Urcuquí has primary and secondary vegetation, paramo, and shrub vegetation (GAD urcuquí, n.d.).

2.2 Field sampling

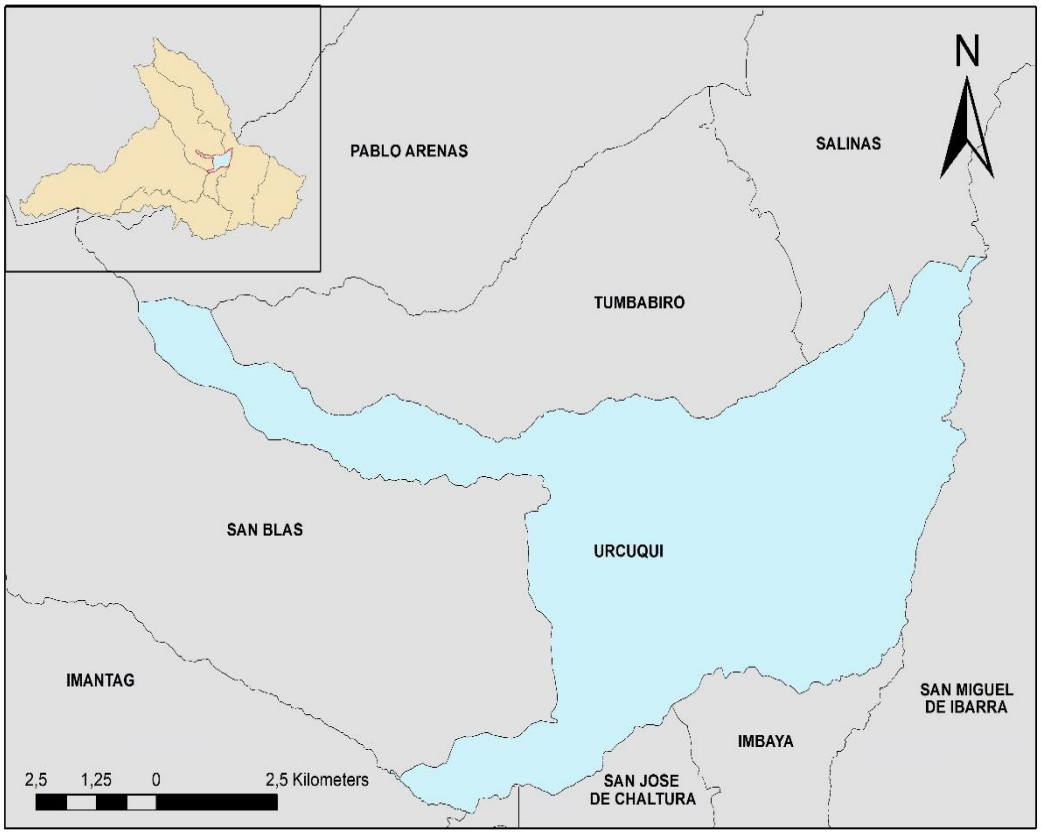

Figure 2: Map of Imbabura province, showing the location of the Canton Urcuquí.

The first samples were collected in February, at the Yachay Tech University near the laboratories of Ingenio for three hours. Three mist nets (Nylon) were used, one 12 m in length by 2,5 m in height, and the other two nests are 6 m long by 2.5 m wide (Figure 3). The nets were opened between 18:00 and 21:00. Every 30 min, the nets were checked to stress from capture. Also, the nets were placed near strategic areas such as water sources (irrigation canal), fruit trees, and areas where there was a higher concentration of vegetation. Bats were caught during free flight (Reiss & Mok, 1979).

The first sampling point had as geographical coordinates 0° 24' 25.932" N, 78° 10' 16.384" W. The second place had as coordinates 0° 24' 25.174" N, 78° 10' 14.867" W. In this area, many palm trees belonging to the Arecaceae family found. Furthermore, grasses and herbs dominated the ground vegetation (Figure 4).

Figure 3: Placement of mist nets in the sampling sites to bats capture in Urcuquí

Figure 4: First sampling area located near Yachay tech laboratories.

Similarly, we continued with the bat sampling, in November 2019 at Yachay Tech University. Here, bats were captured near of Yachay Tech sculptures (Figure 5). This area has more vegetation than in the other two sampling sites, more palm trees of the family Arecaceae, some jasmine trees, and several big trees. In this zone, three mist nets were used: 12 x 2.5 m mist net, and two 6 x 2.5 m mist nets. The second point of sampling has three sites. The geographical coordinates were: first point 0° 24' 16.795" N, 78° 10' 32.281" W, second point 0° 24' 20.602" N, 78° 10' 31.535" W and the last point 0° 24' 25.817" N, 78° 10' 18.075" W. At the second point, the mist net was established near an irrigation canal with had Araceae. Moreover, the third point the irrigation canal.

Figure 5: The second sampling area located near the Yachay tech sculptures

Finally, the last sampling site was located at the Yachay Botanical Garden in November. Three mist nets were placed near water sources and fruit trees. The geographical coordinates were: first point 0° 25' 2.276" N, 78° 11' 18.341" W, second point 0° 25' 2.642" N, 78° 11' 17.825" W, and third point 0° 25' 2.438" N, 78° 11' 16.566" W. This area included the orchid garden path. Avocados, soursop and lemon trees, species of orchids were present.

Saliva samples were collected with sterile cotton swabs rubbing the palate and tongue very carefully (Figure 6 b-c). The bat was allowed to bite the swab for a few minutes to obtain a considerable amount of saliva. Subsequently, the swabs were deposited in 15 ml falcon with 2 ml of PBS buffer (Figure 6d). Next, the

falcon tubes were sealed with parafilm to avoid any contamination and leaking (Figure 6a). Finally, the samples were carried to the laboratory for further analysis.

To identify the species of captured bats, "A field guide for mammals in Ecuador" was used (Tirira, 2017). The measurements (Figure 7a) used by the author are hind foot length (HF), ear length (E), forearm length (FA), and weight (Tirira, 2017). Moreover, all individuals were labeled with the marking technique of small tattoo punch on the wing membrane of each bat (Figure 7b; Bonnaccorso & Smythe, 1972). The method of marking bats with punch-marking consists of punching small holes in the wing membrane (McCulloch, 1986). This tattoo instrument performs the punching in form the numbers. Punch marking generates a white scar tissue forming in 10 days, which lasting up approximately 5 or 6 months, therefore, this technique is used as a short-term marking method (Stonehouse, 1977). Finally, all bats were freed after sampling (Figure 7c).

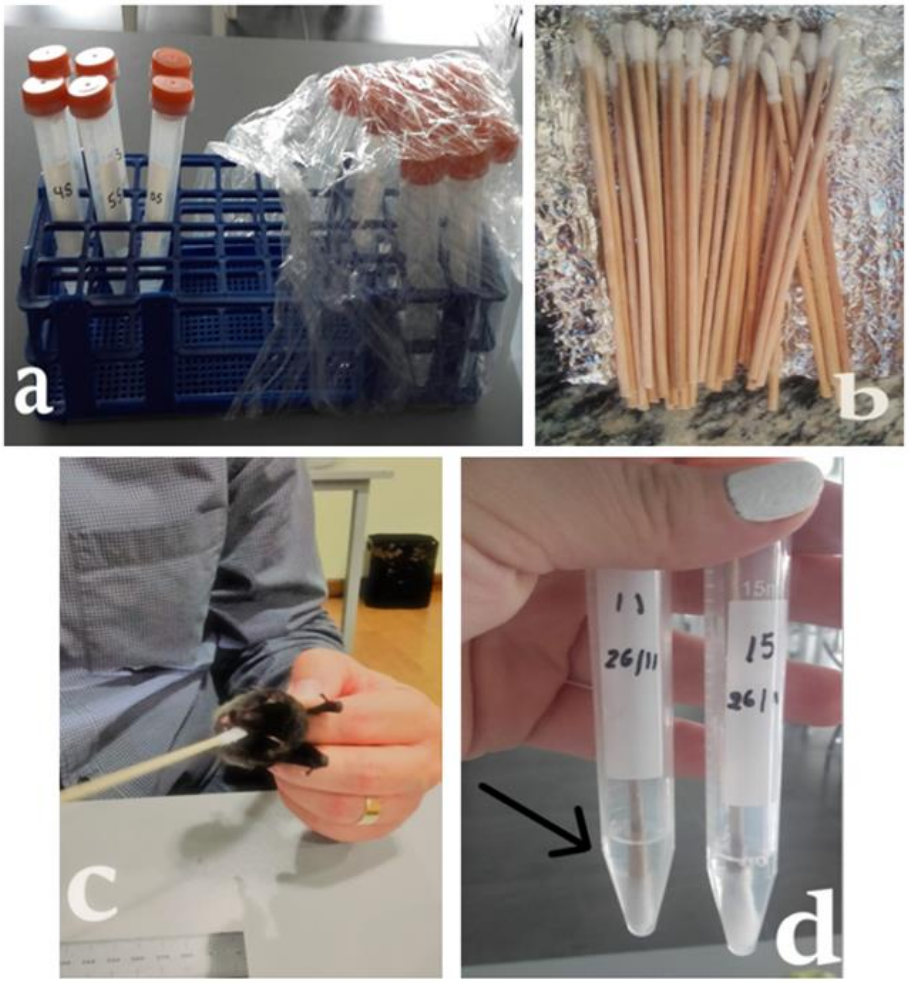

Figure 6: Several aspects of saliva samples (a) Falcon tubes (15 mL) with PBS buffer sealed with parafilm. (b) sterile swabs. (c) collection of bat saliva sample, introducing the swab into the oral cavity, and. (d) storage of swab in PBS buffer to preserve DNA.

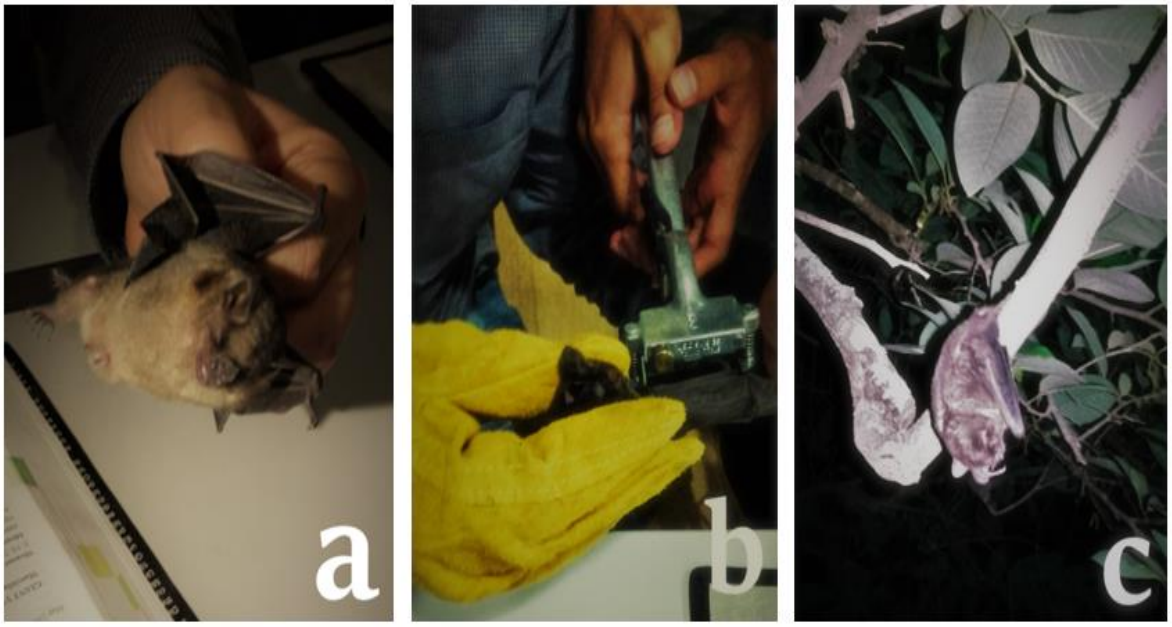

Figure 7: (a) Taking body measurements for species identification. (b) punch-marking used in the left wing of bat. (c) samples and measures, and releasing the bat.

2.3 Sample preparation

Preparation of genomic DNA from bacteria was performed using a modified short protocol of Wilson (2001). Before DNA extraction, the swabs were removed with forceps to avoid contamination of the samples. The reagents used for DNA extraction were; TE buffer, 10% sodium dodecyl sulfate (SDS), 20 mg/ml proteinase K, 5 M NaCl, CTAB/NaCl solution, 24:1 chloroform/isoamyl alcohol, 25:24:1 phenol/chloroform/isoamyl alcohol, isopropanol, 70% ethanol (Wilson, 2001).

First, 1 500 µl of the sample were transferred to labelled microfuge tubes. A sample control was included to detect contaminating DNA. Then, the tubes were spun at maximum speed in a tabletop microcentrifuge for two minutes. Subsequently, the pellet was resuspended in 567 µl TE buffer. Further, 300 µl of 10% SDS were added and 30 µl of 20 mg/ml proteinase K were addded. This preparation was mixed and incubated by 1 hour at 37 °C.

Meanwhile, the CTAB extraction buffer was adapted the protocols of “Cold spring Harbor” Laboratory (2019). The reagents utilized were: 0.03 mg

Polyvinylpyrrolidone (PVP), 300 µl Cetyl trimethyl ammonium bromide (CTAB), 280 µl of 5M NaCl, 40 µl of 0.5M EDTA, 100 µl of 1M Tris-Cl, 2 µl β-Mercaptoethanol, and 248 µl H_2O. Because this buffer is useful only when it is fresh, it was prepared just before use (Cold Spring Harbor Laboratory, 2009). Continuing with the preparation of bacterial genomic DNA, 100 µl NaCl were added to the previous mixture. Next, samples were mixed thoroughly. This step was essential because it produces a CTAB–nucleic acid precipitate. Next, it was added 80 µl CTAB/NaCl solution, which was already prepared previously.

At this point, a slight change in the protocol was made. First, the 680 µl the aqueous phase was transferred to a fresh tube. Later on, to extract the DNA, 700 µl chloroform was placed. Then, this mixture was centrifuged for 5 minutes. Next, 680 µl phenol was extracted of the previous solution, and then tubes were carried to the microcentrifuge for five minutes. It is essential to mention that, phenol-resistant microcentrifuge tubes needed to be used in this step.

In the same way, 600 µl of supernatant were transferred to a fresh tube. Next, to precipitate the nucleic acids, 600 µl of isopropanol were added. At this point, the samples were stored at -4 °C for two days due as a cold microcentrifuge was not available. After that time, the precipitate was washed with 70% ethanol and centrifuged for two minutes. The supernatant was removed, and the pellet was dried in the fume hood. The last step consisted of dissolving the pellet, by adding it to 25 µl TE buffer.

2.4 Nanodrop and PCR

To evaluate the yield and purity of the extracted DNA, 1.5 µl of the samples, were measured in a NanoDrop spectrophotometer. Then, to amplify the 16S RNA gene in the samples, a PCR with 16S RNA primers set was performed. Two types of Taq polymerase were used. For the first reaction; 22 µl PCR SuperMix (Invitrogen), 1 µl forward primer (16S), 1 µl reverse primer (16S) and 1 µl template. For the template samples, the samples with the highest and lowest DNA amounts were used, which were sample # 15 (117.0 ng/µl DNA solution) and sample # 10 (5.7 ng/µl DNA solution). Then, three labeled microfuge tubes received the following mixtures; #1 DNA Control, which was bacterial genomic DNA, #2 DNA low saliva bat, #3 DNA high bat saliva. Subsequently, these tubes

were taken to the thermocycler (Applied Biosystems 9902 Veriti). The final volume was 25 µl. Samples were amplified with the following settings. During stage 1(initial denaturation) mixtures were heated to 94°C for 2 min 13 s. Stage 2 consited 35 cycles consisting of a denaturation step at 94°C for 45 s, annealing step at 50°C for 1 min, and elongation step at 72°C for 1min 30 s. For stage 3 (final elongation) mixtures were heated to 72°C for 10 min, following by cooling at 4°C until removed from the thermocycler. An agarose gel was used to run the DNA samples (tubes #1, 2, 3) with; gel loading dye purple (6x).

In the second PCR test, the same procedure mentioned above was performed, but in this case, 0.2 µl Platinum Taq polymerase (2X, Invitrogen) was used. For the third PCR test it was used; 17 µl H_2O, 2.5 µl of 10x buffer, 2.5 µl of 10x dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl of template and 0.2 µl DreamTaq polymerase (Thermo Scientific^{TM}). Cycles for all PCRs were identical.

3. Results

3.1 Bat samples

A total of 15 saliva samples were collected from the three sampling sites. At the first sampling site during February, no bat was captured. Although there are fruit trees in this area, bats were not active, perhaps due to the high amount of rainfall, and the low. In addition, this area has less natural vegetation than the other sampling sites and it is more open. However, more information is needed to determine if this absence is due to migratory cycles or habitat characteristics. The palm trees were surrounded by a musky odor, suggesting that some animals roost there. No vocalizations were heard during this night.

In the second sampling area, six bats were caught. Three males and three female bats Tirira (2017), two species of the Phyllostomidae family were identified: *Sturnira bogotensis* and *Anoura peruana*. For these samples, a small amount of DNA was obtained (Table 2), which shows the DNA result in the spectrophotometer. The nanodrop revealed a low yield and some impurities. The reason for both observations could be that we did not leave the swab inside the Falcon tube but swirled and discarded it. In addition, these animals may have low load of buccal bacteria.

Finally, nine bats seven males and two females were captured Yachay Botanical Garden, (Figure 2). There was greater difficulty identifying these species because they were quite similar. We classified them as: *Sturnira bogotensis* and *Sturnira bidens*. This time, greater care was taken not to contaminate the swabs. Instead of discarding of swabs, we left them inside the tube that contained the buffer. Table 1 shows the data collected, marking, area, and the identification of each species of bat.

Table 1: Identification of bats according to their morphological characteristics.*

Sample ID	Bat species	Sex	Weight (g)	FA (mm)	E (mm)	T (mm)	HF (mm)	Wing	#WM	Area
1S	*Sturnira bogotensis*	Female	18.7	41.7	16	7	12	Right	0	S.Y
2S	*Sturnira bogotensis*	Male	18.4	42.1	16.5	7	13	Right	1	S.Y
3S	*Anoura peruana*	Male	15.8	45.0	14.5	7	11	Right	2	S.Y
4S	*Sturnira bogotensis*	Male	19.2	43.3	15	6	15	Right	3	S.Y
5S	*Sturnira bogotensis*	Female	19.6	42.9	15	7.5	12	Right	4	S.Y
6S	*Sturnira bogotensis*	Female	21.0	43.7	13	6	13.5	Right	5	S.Y
7S	*Sturnira bogotensis*	Male	18.4	43.1	13.5	6	13	Right	7	Y.B.G
8S	*Sturnira bogotensis*	Female	18.9	45.5	16	7	14	no marking	no marking	Y.B.G
9S	*Sturnira bogotensis*	Male	20.7	43.6	15	7	12	Right	8	Y.B.G
10S	*Sturnira bogotensis*	Male	19.0	42.8	16.5	6.5	12.5	Right	9	Y.B.G
11S	*Sturnira bogotensis*	Male	20.3	43.0	17	6	14	Right	0	Y.B.G
12S	*Sturnira bidens*	Male	15.1	40.3	14	5.5	12	Right	01	Y.B.G
13S	*Sturnira bidens*	Male	17.2	44.8	15	6.5	14	Right	10	Y.B.G

14S	*Sturnira bidens*	Male	19.3	46.5	15	5.5	15	Right	11	Y.B.G
15S	*Sturnira bogotensis*	Female	17.3	43.5	15.5	5.5	14	Right	12	Y.B.G

*Measurements were based Tirira (2017). Weight: Total body mass. FA: Length of the forearm from elbow to base of the thumb. E: Length of the ear from base to tip. HF: Length of the hind foot from the heel to tip of longest claw. Besides, #WM is used to identify the number of marking, T represents the size tragus, and area indicates the place where the bats were captured (S.Y: Sculptures of Yachay Tech and Y.B.G: Yachay botanical garden).

3.2 DNA extraction and PCR

The nanodrop results indicated higher DNA concentration and better purity and yield (Table 2). However, these samples were not ideal.

Table 2: Analysis of DNA bat saliva samples as measured in the Nanodrop spectrophotometer.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	Control	0.4	ng/µl	0.008	0.004	1.99	0.37	DNA	50.00
2	1S	0.1	ng/µl	0.001	0.002	0.71	0.37	DNA	50.00
3	2S	1.2	ng/µl	0.025	0.016	1.54	0.61	DNA	50.00
4	3S	1.9	ng/µl	0.038	0.028	1.33	0.82	DNA	50.00
5	4S	0.8	ng/µl	0.016	0.012	1.28	0.90	DNA	50.00
6	5S	0.4	ng/µl	0.008	0.008	0.94	0.56	DNA	50.00
7	6S	18.8	ng/µl	0.376	0.223	1.68	1.64	DNA	50.00
8	7S	70.5	ng/µl	1.410	0.861	1.64	1.32	DNA	50.00
9	8S	23.9	ng/µl	0.478	0.297	1.61	1.15	DNA	50.00
10	9S	56.7	ng/µl	1.133	0.823	1.38	1.06	DNA	50.00
11	10S	5.7	ng/µl	0.114	0.051	2.22	0.85	DNA	50.00
12	11S	38.0	ng/µl	0.760	0.539	1.41	1.04	DNA	50.00
13	12S	20.5	ng/µl	0.410	0.274	1.49	0.93	DNA	50.00
14	13S	48.9	ng/µl	0.979	0.706	1.39	1.05	DNA	50.00
15	14S	25.3	ng/µl	0.505	0.337	1.5	0.83	DNA	50.00
16	15S	117.0	ng/µl	2.340	1.495	1.57	1.30	DNA	50.00

For the three PCR tests we used three different *Taq* polymerases. Only analysis two yielded amplicons, which could then be used for sequencing or culture methods. For the Taq prepared in the laboratory, the PCR did not show any results (Figure 8a). When commercial platinum *Taq* was used, PCR yielded a sufficiently large amount of DNA in samples 10 and 15 (Figure 8b). Finally, when using Dream *Taq*, obtained poor results. Therefore, future studies should require a good quality polymerase.

Nonetheless, it is important to mention that this investigation only reached until the evaluation and validation of the DNA quality of the bat saliva samples, using spectrophotometry (NanoDrop 2000, Thermo Scientific™) due to limited financing, this investigation cannot continue. Therefore, the following step would be to carry out DNA sequencing to obtain a taxonomic profile of the representative bacteria of the oral microbiota. Despite this, several studies on the results that the sequencing can provide and the identification of microorganisms found will be mentioned.

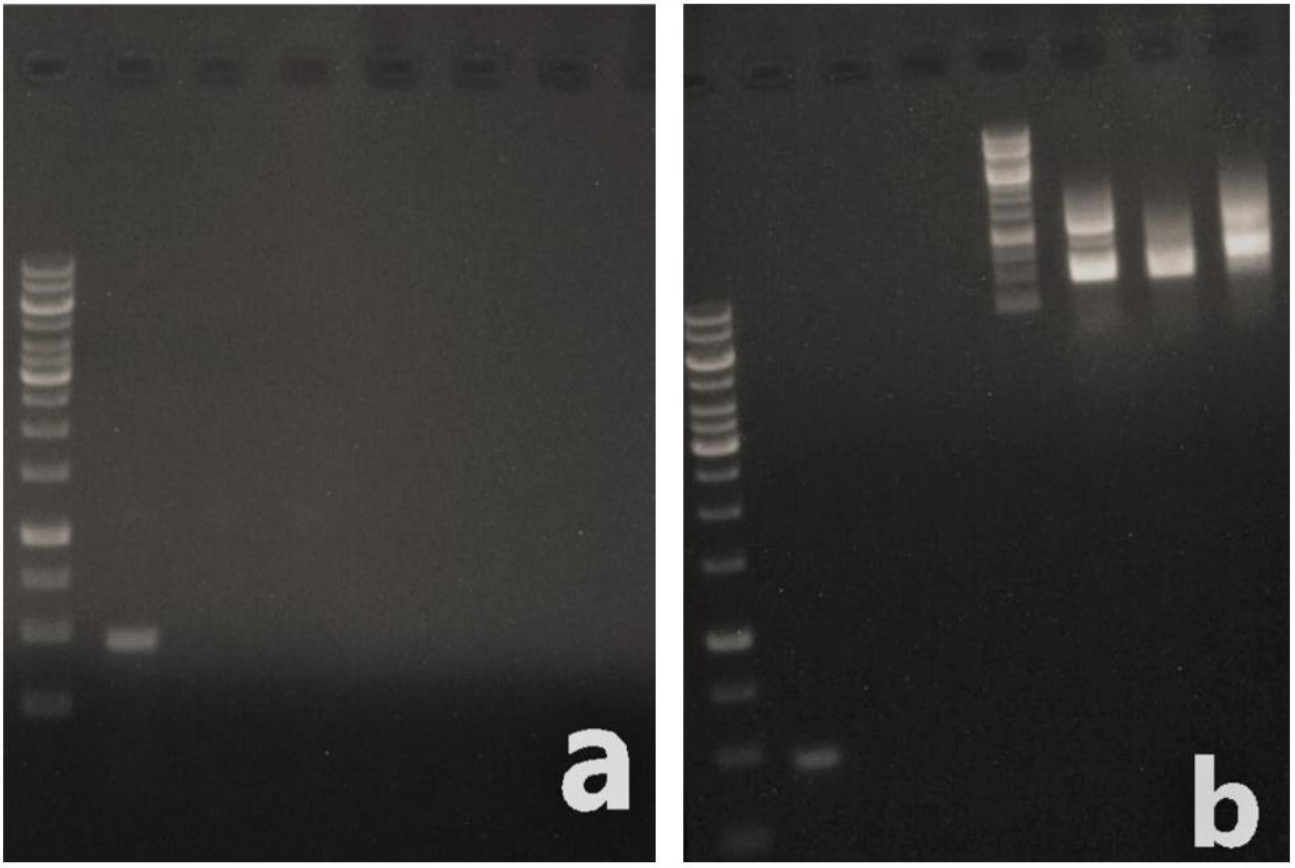

Figure 8: (a) PCR test 1 did not show any amplicons. (b) PCR test 2, which platinum *Taq* was used, amplification was successfully.

4. Discussion

At all sampling sites, we obtained only species from the family Phyllostomidae family, because they are the most diverse both in their ecological niches and in their morphology as well as eating habits that cause them to fly close to the ground. The identification of the species of captured bats was complicated because the morphological characteristics of some species of the Phyllostomidae are very similar. However, with the help of mammalian guide books from Ecuador, the different species of bats were identified, which are shown in table 1. In addition, of a total of 15 captured species, 73.33% *were Sturnira bogotensis*, 20% were *Sturnira bidens*, and only 6.67% *were Anoura peruana*. The common name of the species *S. bogotensis* is Bogotá Yellow-shouldered Bat. In Ecuador, this species is distributed in the Sierra, subtropical, and temperate forests (Romero et al., 2018). In contrast, the common name of *S. bidens* is Bidentate Yellow-shouldered Bat. They inhabit altered primary and secondary forests. The genus *Sturnira* is characterized by having fruit bats that are efficient seed dispersers. Members of this genus have yellowish spots on the shoulders. Ecuador presents 14 genres of *Sturnira* (Romero et al., 2018). Finally, the common name for *A. peruana* is Peruvian Tailless Bat. They feed on pollen and nectar, and sometimes of insects found in flowers. Hence, they are nectarivorous bats that are characterized by elongated snout and tongue. Therefore, Phyllostomidae is the most diverse family among the neotropical bats (Boada et al., 2018).

Genomic DNA extraction for the first six samples, yielded poor results. Only one sample contain sufficient DNA. Genomic DNA extraction was for the following nine samples, produced better results as determined by the Nanodrop. This result could have been a consequence of having let the bats bite the swabs for a longer period. Another important factor could be that this time the swabs was were left in the buffer and stirred for a longer time, to have the highest concentration of cells. Even so, a good yield, purity, and DNA were not obtained. Thus, the technique for taking saliva samples has to be improved. In addition, another type of buffer and using a DNA extraction kit to eliminate the highest amount of contamination should be considered.

To verify that there was enough DNA, three different PCR reactions were performed. First, *Taq* polymerase was used with an internet protocol. No

amplification was observed (Figure 8a). Then, a commercial Platinum *Taq* was used, resulting in higher and lower amounts of DNA (samples # 10 and # 15 respectively). Here the results show that DNA was amplified (Figure 8b). Finally, the last PCR test was performed for the same two previous samples, but when using Dream *Taq* polymerase, no results were obtained. Thus, it is recommended to use a good quality *Taq* polymerase to continue with the DNA amplification process.

On the other hand, to characterize and identify microorganisms, I considered using microbiological techniques such as cell cultures. However, in order not to lose information on the bacterial population in this microhabita, no culture method was performed because it would probably favor the growth of some bacteria and not others. An enrichment media that favors the entire community of bacteria representative of the oral cavity is required. Therefore, methods carried out by various investigations that worked with saliva samples from either bat or other animals will be mentioned. For instance, I suggest to replicate the method of culture applied by Galicia et al. (2014) whose used 5g of meat peptone, 4g of lactose, and 10g/100mL of calcium carbonate as enrichment medium. Then, they used a simple streak plate in differential, selective, and chromogenic media at temperature between 30 °C and 45 °C. For the identification of bacterial colonies, they used Gram staining, presence of spores, and several biochemical tests. After performing statistical analyses, they determined 26 species of bacteria; including Firmicutes and Proteobacteria. Finally, they found that there are differences bacterial diversity between the anal and oral cavities of frugivorous bats. In addition, when they analyzed the bacterial communities of different species of bats (Frugivorous and hematophagous), they found similarities in microorganisms. However, they find exclusive bacteria to the oral cavity, such as *Bacillus cereus* for nectarivorous, *Aeromonas hydrophyla, Staphylococcus aureus*, and *Serratia marcescens* for hematophagous and *Pseudomonas aeruginosa* for frugivorous. However, comparing the communities of bacteria among many bat species, it can be presumed that firmicutes and proteobacteria are part of the healthy intestinal microbiota of bats (Galicia et al., 2014). Information on the bacterial activity of the salivary microbiota, however, is limited (Belstrøm et al., 2017). Other investigation showed that the bat species which share an ecological niche, have the same strains of *Bartonella* (Mühldorfer, 2013).

Therefore, it is necessary to create microbiological methods to characterize these bacteria and find a correlation with the health and disease of bats.

Another technique for the identification of bacteria is sequencing. Neverthelless, due to limited financing, it could not be done. Many investigations use independent cultures to know the interactions between microorganisms and microhabitats within animals, such as PCR-amplified marker genes (Salter et al., 2014). Also, current advances using metagenomic sequencing allow for rapid screen of the microbiomes of bats from saliva and urine (Dietrich et al., 2017). For example, Newman et al. (2018) used Hot StarTaq Master Mix Kit to amplify the 16S rRNA gene. Then, to create the library, they used Illumina DNA sequencing (Newman et al., 2018).

In similar way, in another investigation, they also used Illumina sequencing of 16S amplicons to construct NGS libraries. Moreover, they studied the dynamics of bacterial and viruses using linear models. In the phylogenetic analysis of saliva, they found that fruit bats were infected with *betaherpesvirus* and *gammaherpesvirus.* Further, they found a relationship between the sex of bats and the composition of the microbiota, in which males revealed most phylotypes. Presumably as a result of the steroid/endocrine differences and feeding behaviors between females and males bats. Another result was the difference between pregnant and non-pregnant females. For instance, the reproductive condition revealed that pregnant bats have *Actinobacillus* and *Streptococcus.* Therefore, reproduction and sex of bats do influence the composition of the oral microflora (Dietrich et al., 2017). Mühldorfer (2012) suggests that the eating habits of bats will influence even the presence of enteropathogens such as *Shigella, Campylobacter, Yersinia*, and *Salmonella*. Nevertheless, they could acquire some of these bacteria from the roost places or livestock environments.

Similarly, a study conducted of the oral cavity in humans used metagenomic and transcriptomic analysis. They researchers have shown three more abundant bacterial genera. These are *Streptococcus, Prevotella*, and *Veillonella.* Also, to determine the microbial activity, they used the DNA to RNA ratio, for which, the phylum Firmicutes expressed a positive ratio in all groups. In this study, they used 2100 Bioanalyzer to quantify, residual, and quantity DNA and RNA. To prepare the library, they used TruSeq Stranded mRNA protocol, and for taxonomy, they used phylogenetic analysis (Belstrøm et al., 2017).

In a different study, Sturgeon et al. (2014) conducted an oral microbiota study in cats. They used Illumina MiSeq to sequence and OTUs (operational taxonomic units) to calculate bacterial diversity. In this investigation, it was found that the most abundant species were; Proteobacteria, Bacteroidetes, Firmicutes, Spirochaetes, Fusobacteria, and Actinobacteria. Moreover, they suggest that the oral microbiome is more diverse than the intestinal microbiome of cats and other animals (Sturgeon et al., 2014). Despite this fact, Dietrich et al. (2017) mention that urine contains more diversity than saliva and feces. This result suggests that the urine has its microbiota and is not sterilized (Dietrich et al., 2017) as in the opposite case of saliva that is sterile when it is in the oral cavity, but when a sample is taken, the saliva has a different microbiota (Belstrøm et al., 2017). Unlike humans, there is less diversity in the oral cavity than in another microbiome (Sturgeon et al., 2014).

Once the bacterial populations have been characterized, we can talk about the specificity of bacteria found in different bat species. For example, Galicia et al. (2014) mention that although *Bacillus cereus* causes food poisoning, it also plays an ecological role in the degradation of xylan and cellulose. Therefore, this bacterium could help in the digestion of these polysaccharides. Another bacterium that was found is *Xanthomonas*, which is a phytopathogen. Bats can contribute to their distribution as dispersers of this and other pathogen. In adittion, *Aeromonas hidrophyla* bacteria, found in haematophagous bats can cause tissue damage. However, in bats, it allows them to digest red blood cells. Finally, a pathogen of both humans and other animals that causes cutaneous infections and can even lead to death is *Staphylococcus aureus*. This bacterium produces exoenzymes that activate a glycoprotein whose function is to dissolve clots. This would help hematophagous bats in their diet (Galicia et al., 2014).

Finally, as can be seen in the studies mentioned above, some researchers commonly use Illumina sequencing to characterize samples as feces, urine, or saliva. In this way, they obtain a complex bacterial system or microbiome profiling of the oral cavity. Then, they perform statistical and bioinformatic analyses to organize, interpret, and present the information collected.

Finally, it can be seen that non-cultural methods can describe the general bacterial diversity found in the oral microbiome of several animals and humans. Despite being more expensive methods, the results are excellent. On the other

hand, statistical analyses can also be used to determine which bacteria can be considered part of the healthy biota of the oral cavity. Also, positive results can be obtained from low DNA samples and metagenomics tools. The role of oral microbiota in animals is not only important for determining which diseases can affect humans and animals. This knowledge can also help to conserve bats and the many ecological services in all parts of the world.

5. Conclusions and Recommedations

- The analysis of oral microbiota in bats could be used as a starting point to understand the patterns of evolution that occur in different animals groups. Therefore, bats can be used as models to understand the interactions between microorganisms, physiology, and ecology.
- Diet, sex, age, reproductive status, ability to flight, and ecological niches can influence the microbial composition of saliva. That is, there is a correlation between the ecology of bats and bacterial communities in their different microbiomes.
- There is great variety in the composition of the oral microbiota of different bats species due to their eating habits. In the same way, similarities were also found in the microbiota various species of bats, which indicates that these bacterial populations are part of the healthy biota of these mammals.
- Extrinsic factors such as environmental stress and human activity, and intrinsic factors such as sex, age, social status influence the susceptibility of contracting more bacteria and increase their transmission.
- DNA sequencing provides researchers with a list of bacterial populations. However, to understand the function of these bacteria, they must be known as living organism.
- More research is needed to create cultivation-dependent techniques as they are still limited due to a lack of understanding in this methodology.
- For future studies, it is recommended to analyze bacterial communities focusing on the following excretion routes: urine, feces, and saliva. This recommendation could help to understand the microbiota of bats and their relationship with the transmission of infections or pathogenic bacteria.

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