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Urbanisation and Altitude impact gut microbiome diversity in an Andean Frog

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Urbanisation and Altitude impact gut microbiome diversity in an Andean Frog

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RESUMEN

El bienestar de un ser vivo depende de un grupo de variables entre estas la funcionalidad de los microorganismos que sobreviven en su interior. El conjunto de estos microorganismos se lo conoce como microbioma, y en estudios recientes se ha determinado que su composición y rol puede ser modificado por cambios en los nichos ecológicos del huésped. Sin embargo, los estudios de microbioma en anfibios son escasos y la mayoría se ha concentrado en el microbioma de la piel. Además el estudio del microbioma intestinal puede aportar conocimiento sobre el efecto de los impactos antropogénicos en las poblaciones de anfibios. *Pristimantis unistrigatus* es una especie de anfibios que está distribuida entre los 2200 y los 3400 msnm y habita en una variedad de nichos ecológicos, desde vegetación nativa hasta jardines urbanos. Mediante la secuenciación de nueva generación Ilumina MiSeq de las zona variables V3 y V4 de los fragmentos del gen bacteriano 16S ARN ribosomal se caracterizó la composición del microbioma intestinal de *P. unistrigatus*, de 32 individuos a lo largo de 4 localidades con un nivel distinto de desarrollo urbano y una altitud diferente en el Valle de Quito. Los análisis de bioinformática se realizaron con Qiime2, y demostraron que la abundancia relativa de bacterias era significativamente diferente entre los distintos grupos. Clostridiales son proporcionalmente más abundantes en las localidades rurales y bajas. Bacteroidaceae, Erysipelotrichaceae, Desulfovibrionaceae, Enterobacteriaceae, Bacteroidaceae y Lachnospiraceae son más abundantes en las localidades altas. Lachnospiraceae, Ruminococcaceae y Erysipelotrichaceae son más abundantes en las localidades urbanas. Cada población de anfibios presento una abundancia distinta entre los diferentes grupos de bacterias, demostrando que las variables de desarrollo urbano y altitud si modificaron a la composición del microbioma intestinal.

Palabras clave: microbioma intestinal, desarrollo urbano, altitud, anfibios, Quito, impacto antropogénico, secuenciación de nueva generación.

ABSTRACT

The welfare of a living being depends on a group of variables among these the functionality of the microorganisms that survive inward the host. The agregation of these microorganisms is known as microbiome, and recent studies have determined that their composition and role can be modified by changes in the ecological niches of their host. However, studies of microbiomes in amphibians are scarce and most have focused on the skin microbiome. In addition, the study of the intestinal microbiome can provide knowledge on the effect of anthropogenic impacts on amphibian populations. *Pristimantis unistrigatus* is a species of amphibians that is distributed between 2200 and 3400 masl and lives in a variety of ecological niches, from native vegetation to urban gardens. Through the new generation sequencing Ilumina MiSeq of the variable zones V3 and V4 of the fragments of the bacterial gene 16S ribosomal RNA we characterized the composition of the intestinal microbiome of *P. unistrigatus*, of 32 individuals throughout 4 locations with a different level of urban development and a different altitude in the Valley of Quito. The bioinformatics analyzes were performed with Qiime2, and showed that the relative abundance of bacteria was significantly different between the different groups. Clostridiales are proportionally more abundant in rural and low locations. Bacteroidaceae, Erysipelotrichaceae, Desulfovibrionaceae, Enterobacteriaceae, Bacteroidaceae and Lachnospiraceae are more abundant in high locations. Lachnospiraceae, Ruminococcaceae and Erysipelotrichaceae are more abundant in urban locations. Each amphibian population presented a different abundance among the different groups of bacteria, evidencing that the variables of urban development and altitude did modify the composition of the intestinal microbiome.

Key words: intestinal microbiome, urban development, altitude, amphibians, Quito, anthropogenic impact, new generation sequencing.

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URBANISATION AND ALTITUDE IMPACT GUT MICROBIOME DIVERSITY IN AN ANDEAN FROG

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Introduction

Microorganisms, primarily bacteria, but also fungi and viruses, have developed complex relationships with bigger organisms, such as mammals, fishes, amphibians, arthropods, among others. Recent studies have shown that play an essential role in disease resistance, host health and adaptation to biotic and abiotic stressors (Jimenez & Sommer, 2017; Colston & Jackson, 2016; Chang et al., 2016; Bahrndorff et al., 2016). Also, showing a mutualistic, commensal, symbiotic or pathogenic relationship between the microbial community and their host (Jimenez & Sommer, 2017; Karl et al., 2018). The number of bacterial cells exceed the host's cells in at least 10 times, but regardless the number of cells, the convoluted communities, defined as microbiome is what makes so important their understanding (Colston & Jackson, 2016). Research advances in this area has allowed a deeper comprehension of the microbial diversity in any sample of interest, primarily by the sequencing of fragments of the bacterial 16S rRNA gene. And thanks to the development of next generation sequencing (NGS) the characterization of this microbial communities is faster and more affordable (Colston & Jackson, 2016; Bahrndorff et al., 2016; Jimenez & Sommer, 2017).

The study of microbiomes has been focused mostly on humans, since the Human Microbiome Project (HMP) started in 2008. Especially when talking about gut microbiome, where research has concentrated in mammals, particularly humans. Although, this opened a number of questions about how the gut microbiome functions in other animals. The animal microbiome has evolved with their hosts, being shaped be their genotype, life stage and the ecological and physiological conditions (Bahrndorff et al., 2016). And in turn, the microbiome assists in the host's nutrient acquisition, immune response (Mashoof et al., 2013; Colombo et al., 2015), behavior (Banas et al., 1988), development (Knutie et al., 2017a; Warne et al, 2017; Chai et al. 2018)., reproduction and most important host's health (Colston & Jackson, 2016; Chang et al., 2016; Warne et al, 2017; Pereira et al., 2015). Any alterations of the microbiome composition could trouble its normal capabilities, turning the host more vulnerable to the effect of unfavorable environmental conditions (Jimenez & Sommer, 2017; Zhang et al., 2016; Mu et al., 2018; Kohl et al., 2014; Huang et al. 2018).

The gut microbiome is the primary symbiotic relationship in the host´s organism, and it is involved with the capture of nutrient for the host´s metabolism (Sugita et al. 1984; Chang et al., 2016; Warne et al, 2017), fermentation of fiber and synthesis of essential amino acids (Kohl et al., 2014). The importance of these relationships has been just recently considered for the success of certain species in their environment (Kohl et al., 2014; Warne et al, 2017; Huang et al., 2018), and recent studies have shown its implications for wildlife conservation (Jimenez & Sommer, 2017; Bahrndorff et al., 2016). However, despite the expansion in this area of research, most of the information comes from data in mammals. The number of studies in amphibians is not so significant and notwithstanding, the majority of these studies are from animals kept in confinement (Warne et al, 2017; Benno et al., 1992) being the resulted information useless for wild

animals researches (Colston & Jackson, 2016) due to all the environmental disrupters (Knutie et al., 2017b) that are not always taken into account when keeping toads in captivity (Smith & Stoskopf, 2007).

The growth of human manipulation in the environment and the warming of the ecosystems has caused changes in species abundance and distribution, especially in amphibians (Pounds et al., 1999). Declining in the amphibian population has been reported since 1980s all over the world, a key region is the tropical Pacific Ocean (where Ecuador is also comprehended), where the conditions have become warmer since 1970s (Pounds, 2001). Temperature and moisture are the two components in climate change that have affected directly the amphibian biology (Carey et al., 2003) changing body physiology there by affecting the gut microbiome (Amato et al. 2013).

In amphibians between 1990 and 2016, there were only 21 studies of microbiome in wildlife frogs, from which only 5 used NGS (Colston & Jackson, 2016) but none of them were made in Ecuador, whereas mentioned is a conflicted area. Ecuador is the fourth most diverse in amphibian fauna with 609 species (558 of the order Anura) and the gender *Pristimantis* is by far the most abundant (BioWeb, 2019). *Pristimantis unistrigatus* is a common species inside this group, is a small frog that occurs from 2200 to 3400 masl in the south of Colombia to the center of Ecuador. Across this elevation range, vegetation changes from forested regions in sub-temperate wet and humid temperate regimes to pastures, ditches, shrubs, crops, forest edges and urban areas. *P. unistrigatus* is tolerant to human disturbance and therefore is commonly found in urban areas and farmlands. It is a common terrestrial nocturnal species occurring in leaf litter. A number of characteristics has allowed this species to be found in different ecosystems with a variation in the grade of human disruption. Between the evolution and adaptation of this organism, the gut microbiome could be playing a crucial role. Then the focus of this study

is to determine if the composition of the microbial communities within this species has change across 4 niches with different altitude and a different level of human impact.

Material and Methods

Sample collection.

Forty male frog samples (10 from each location) of *Pristimantis unistrigatus* were collected at the Metropolitan District of Quito, from the rural areas: Hacienda Sierra Alisos (0.408628W 78.589187S, 3200 masl, HAP) and Hacienda San Francisco (0.441422W 78.561351S, 2830 masl, HCM) and from the urban areas: Urbanización San Martin (0.177726W 78.496875S, 2880 masl, SMQ) and Urbanización La Colina (0.317348W 78.434793S, 2510 masl, VH) from January to May, 2018 (Figure 13).

P. unistrigatus males have a characteristic chant to woo the female, helped by this during the nocturnal field trips the samples were collected. When an individual was spotted before capture, the corporal temperature was measured with the help of an infrared thermometer. Then with surgical globes the individual was captured, for the microbiological composition would not be compromised. The spots of collection were located with marking tape, and there was measured soil, substrate, and environmental temperature, environmental humidity, and wind velocity. Prior to the sacrifice of the frog, it was washed with distilled water for 30 seconds in ventral and dorsal position. With a sterile swab took a sample of the abdomen and with another swab a sample of the back, this were stored in sterile falcon tubes of 5ml.

Each individual was sacrificed at the field, with an 8kq CO2 tank. The frog was placed inside an Erlenmeyer that was connected to the CO2 tank. The CO2 flowed through the sealed Erlenmeyer for 10 seconds, and then the valve was closed. The frog remained inside the Erlenmeyer for other 10 to 30 seconds, until the frog fainted. Then

the individual is extracted from the Erlenmeyer and its reflexes were checked. With the help of a swab was checked if the blinking reflex disappeared and the absence of the reflex of recovering the ventral position when positioned backwards.

From there, proceeded with the extraction of the tissues. For the dissection, the frog was positioned in a dorsal position and open with an "H" form cut. The digestive system was extracted, then the stomach and intestine are separated and stored into two different sterile falcon tubes of 5ml. All the samples were transported and brought to San Francisco de Quito University (USFQ) to a freezer at -80 °C.

Diet analysis.

The stomach sample was defrosted at the Zoology Laboratory of the USFQ. All the content of each stomach was extracted, the preys were identified to order level under a stereomicroscope (Hyslop, 1980). Of each prey the length and width were measured to calculate the importance of each prey, with the formula IRI= O% (N% + V%), where O%, N%, V% are the percentage of occurrence, relative abundance, and measured volume of each prey category, respectively in every stomach (Chang et al., 2016). Where the volume was calculated with the formula:

$$V = \frac{4}{3\pi(C)(L^2)}$$

Where C is half of the length and L is half of the width. To estimate the food and spatial niche breadth between habitats was used the Simpson's index of diversity (B), with the following formulas:

$$D = \sum \left(\frac{n}{N}\right)^2 \qquad \text{B=1-D}$$

Where *n* is the total of organisms of a determined species and N is the total number of organisms of all species. Measured between 0 and 1, where 1 is the most diverse (Diaz & Rocha, 2007).

To estimate the overlap between the four locations was used the coefficient of symmetry of overlapping, was used the next formula:

$$O_{jk}=\frac{\sum_{i=1}^{n} pij \times pik}{\sqrt{\sum_{i=1}^{n} pij^2 \sum_{i=1}^{n} pik^2}}$$

Where 0 means there's no overlap and 1 means complete overlap (Diaz & Rocha, 2007). The number of prey items, relative of abundance and prey volume was analyzed with MiniTab (2018) with a T test to determine differences between the urban and rural habitats and the higher and lower habitats.

Intestinal microbiome.

The intestinal microbiome of 32 frogs were analyzed (8 from each location), of the 40 collected due to budget and DNA concentration constraints. Intestinal microbial DNA was extracted with PureLink Microbiome DNA Purification Kit (Stool Samples). For each sample, we amplified the V3 and V4 hypervariable 16S rRNA region using the primer set Bakt_341F: CCTACGGGNGGCWGCAG and Bakt_805R: GACTACHVGGGTATCTAATCC. Sequencing on Miseq 300bp PE at Macrogen Inc. (2018) (Song et al., 2018). The bioinformatic analysis were performed using Qiime2 2018.4 version, every sequence was assigned to a barcode, we demultiplex the sequences and determine how many sequences were obtained per sample and their quality. For the denoising and control process of the sequences, DADA2 was used to detect and correct the sequenced data (Mashoof et al., 2013).

The Alpha diversity analysis that were estimated were Shannon's diversity index (Medina et al., 2017), Observed OTUs (Chai et al., 2018), Faith's Phylogenetic Diversity (Bletz et al., 2016) and Pielou's Evenness (Knutie et al., 2017b), these metrics were computed for each frog with Qiime2. On the other hand, for the Beta diversity we calculated Unweighted and Weighted UniFrac distances between samples in Qiime2 (Knutie et al., 2017a). The sequencing depth for the analysis was 2823 sequences. This value was chosen based on the number of sequences in the LBI012 sample. The visualizations resulting from the prior analysis were generate with the Emperor tool (Chai et al., 2018).

Results

Diet differentiation between habitats.

Urban vs. Rural.

A total of 32 *P. unistrigatus* individuals, from which 17 came from a rural habitats (HAP and HCM) and 15 from urban habitats (VH and SMQ) were analyzed. A total of 133 individual prey items were identified to 11 orders (Table 1). In all habitats Ixodida had the higher index of relative importance (IRI) score (147261,99). Followed with the highest scores in rural habitats by Diptera (17114,23) and urban habitats by Coleoptera (15183,6) (Table 1, Figure 1). The number of prey items (p=0,896), relative abundance (p=1,00) and prey volume (p=0,682) were not significantly different between the four locations (Table 2). But according to the Simpson's Index of Diversity the urban habitats (B=0,818) were by little more diverse than the rural habitats (B=0,729) and the overlapping between the urban and rural habitats was O=0,864 (Table 3). Lepidoptera, Neuroptera, Dermaptera, Isopoda and Harpacticoida were only present in the urban habitats (Table 1).

High vs. Low.

A total of 32 *P. unistrigatus*, from which 15 came from the higher locations (HAP and SMQ) and 17 from the lower locations (HCM and VH) were analyzed. In all In all habitats Ixodida had the higher index of relative importance (IRI) score (147261,99). Followed with the highest scores in the higher locations by Aranae (43237,45) and in the lower location by Coleoptera (61682,71) (Table 1, Figure 2). The number of prey items (p=0,400), relative abundance (p=1,00) and prey volume (p=0,955) were not significantly different between the higher and lower habitats (Table 2). But according to the Simpson's Index of Diversity the lower habitats (B=0,803) were by little more diverse than the higher habitats (B=0,751) and the overlapping between the higher and lower habitats was almost complete with O=0,951. Lepidoptera, Neuroptera, Dermaptera and Harpacticoida were only present in the lower habitats (Table 1).

Composition of the intestinal microbiome.

Gut bacterial communities were investigated using 16S rRNA gene in 31 individuals of *P. unistrigatus* at Quito. The sequencing resulted in a total of 364 243 high-quality sequences from all the samples. Multiple rarefaction curves generated from the observed OTUs reached a plateau phase suggesting that high sampling coverage were achieved in all samples (Figure 3). From the taxonomic analysis a diverse community structure was revealed, dominated by members of the phyla Firmicutes (mostly Clostridia), Proteobacteria (mostly Gammaproteobacteria) and Bacteroidetes (mostly Bacteroidia) (Figure 4). The concentration of certain groups of bacteria was different between the four locations (Figure 5), suggesting a different microbiome composition among the four population of Andean frogs.

The alpha diversity (faith and evenness) of intestinal microbiome was estimated by Kruskal-Wallis. The faith between all groups was not significant (*P*=0.499), as

consequence the richness of species in every location was not different and the pairwise analysis showed no significant *P*-values in any of the groups (Table 4). Nevertheless, the evenness of the intestinal microbial community was different between all groups (*P*=0.009), showing the species of bacteria inside each group were different among them (Table 4). The pairwise analysis showed significant differences between HAP-HCM (*P*=0.012), HAP-SMQ (*P*=0.015), and HCM-VH (*P*=0.022); but there were no differences between HAP-VH (*P*=0.643), HCM-SMQ (*P*=0.100) and SMQ-VH (*P*=0.084) (Table 4).

The beta diversity measures the diversity of species between the intestinal microbial communities of each group. Based on weighted UniFrac distance with PCoA analysis (Figure 6) there isn't a defined cluster for any of the groups but there is a tendency for each individual of the group to be close to each other, there were significant differences in the pairwise PERMANOVA results with 999 permutations between HAP-HCM (*P*=0.036), HAP-SMQ (*P*=0.010), HCM-VH (*P*=0.008) and SMQ-VH (*P*=0.006), while between HAP-VH (*P*=0.173) and HCM-SMQ (*P*=0.140) there were not significant differences (Table 5). On the other hand, the PCoA analysis based on the unweighted UniFrac distance (Figure 7) showed no define clusters within the groups, in the pairwise PERMANOVA results there were significant differences between HAP-HCM (*P*=0.044), HAP-SMQ (*P*=0.012), HAP-VH (*P*=0.006), HCM-VH (*P*=0.004) and SMQ-VH (*P*=0.012), while there were not significant differences between HCM-SMQ (*P*=0.619) (Table 5). The PCoA analysis based on the Jaccard distance shows 2 clusters (HAP and VH), while the other two groups were disperse through the entire graphic (Figure 8).

Differential abundance analysis using balances in gneiss revealed that proportions of *Clostridiales* were a lot higher in the lower and rural locations (Figure 9; Figure 10).

Bacteroidaceae, Erysipelotrichaceae, Desulfovibrionaceae, Enterobacteriaceae, Bacteroidaceae and Lachnospiraceae were more present in the higher locations (Figure 9). Lachnospiraceae, Ruminococcaceae and Erysipelotrichaceae were more present in urban locations (Figure 10).

Microbiome modifiers.

Soil temperature.

In PERMANOVA results based on the weighted UniFrac distance (permutations=999) showed significant differences between VH and SMQ at the range of temperatures 14-15°C vs. 15-16°C (P=0.047) (Table 6) while on the other pair of ranges there were no significant differences. Between VH and HCM at the range of temperatures 13-14°C vs. 14-15°C (P=0.032) and 14-15°C vs. 15-16°C (P=0.030) (Table 6), while on the other pair of ranges there were no significant differences. Additionally, comparisons between all the other groups there were no significant differences among the ranges of temperatures.

On the other hand, in the PERMANOVA results based on the unweighted UniFrac distance (permutations=999) between VH and HCM showed significant differences at the range of temperatures 14-15°C vs. 15-16°C (P=0.029) (Table 7), while on the other pair of ranges there were no significant differences. Between SMQ and HCM at the range of temperatures 12-13°C vs. 15-16°C (P=0.036), and 14-15°C vs. 15-16°C (P=0.040) (Table 7), while on the other pair of ranges there were no significant differences. Between all the other groups there were no significant differences among the ranges of temperatures.

In all the pair of locations the soil temperature seemed to be a factor of influence in the composition of the gut microbiome, but the PCoA analysis (Figure 11) showed a

correlation that every location had a specific soil temperature, so taking all of this into consideration the soil temperature was a variable of confusion, but did not restructure the microbial community.

Relative environmental humidity.

The PERMANOVA results based on the weighted UniFrac distance (permutations=999) showed significant differences between SMQ and HAP at the humidity ranges 90-93% vs. 94-97% (P=0.025) and 90-93% vs. 98-100% (P=0.029) (Table 8), while on the other pair of ranges there were no significant differences. Between HAP and HCM at the humidity range 90-93% vs. 98-100% (P=0.019) (Table 8). Between all the other groups there were no significant differences among the humidity ranges. Moreover, the PERMANOVA results based on the unweighted UniFrac distance (permutations=999) showed significant differences between SMQ and HAP at the humidity ranges 90-93% vs. 94-97% (P=0.037) and 90-93% vs. 98-100% (P=0.020) (Table 9), while on the other pair of ranges there were no significant differences. Between all the other groups there were no significant differences among the humidity ranges.

Location.

Differential abundance analysis using balances in gneiss revealed that proportions of *Clostridiales* were a lot higher in HCM in comparation to SMQ, while Lachnospiraceae, Eubacteriaceae, Phyllobacteriaceae and Intrasporangiaceae were more abundant in SMQ than in HCM (Figure 11). Even though *Bacteroides*, and *Clostridium*, are present in HAP and VH, the proportion in which certain species are present in one of the two locations is completely different (Figure 12). *Parabacteroides* and Veillonellaceae are more present in VH, while Ruminococcaceae is more present in HAP (Figure 12).

Discussion

In the present study, we used four locations to determine if the level of urbanization and the altitude would be a factor that had a potential role in changes in the structure of gut microbiome of *P. unistrigatus*. The factors influencing the association of microbial communities inside a host are the primary interest in microbial ecology, which can be diet, physiological conditions and the host species (Sugita et al., 1985). This host-microbiome symbiosis may facilitate the survival rate of a species facing the rapid ecosystem changes around the globe (Bletz et al., 2016). The dominance of certain groups of bacteria can be beneficial, while the presence of other groups can be harmful for the frog (Karl et al., 2016). Is so that the study of the composition of the intestinal microbiome of bigger organisms may be an insight of the state and evolutionary story of a certain population.

Diet structure and changes can be one of the external factors that alter the composition of the intestinal microbiome. Is so that frogs gut microbiome can be influenced by soil microorganisms due to the ingestion of the preys covered in soil bacteria found in their actual niche (Huang et al., 2018). The lack of differences in stomach contents of Andean frogs between urban vs. rural and high vs. low habitats may reflect that. However even with the human impacts there is not a significant disturbance to alter the faunal species composition. The diet analysis showed that the food volume, number of prey items and relative abundance of prey categories were not significantly different between the four locations.

The high prevalence of Ixodida, can be a reflection of the presence of domestic animals, cattle in rural habitats and mascots in urban habitats. This domestic animals are again the reflection of the expansion of the human impact. The environment is changing and a lot of habitats are been destroyed, so prey resources also change (Chang et al. 2016).

The diverse degree of human impact among all the groups suggested that the diet would have changed between the individuals. But the absence of statistical differences and the high overlap of species between the groups showed that the dietary tendency of the frogs was the same among all groups, meaning this couldn't be a factor of influence to the differences in the composition of the gut microbiome.

The structure of the gut microbial communities are highly determined by environmental factors of the niche were the host develops (Chang et al. 2016). The adaptation to certain ecosystems can change the hosts tolerance, behavior and interaction with its surroundings, which can alter the gut microbiome (Huang et al., 2018). The amphibians' intestinal microbiome is similar to that of mammals and birds. And like that it can vary due to external factors (Benno et al., 1992). The intestinal microbiome analysis showed a clear variation throughout the various locations, suggesting the environmental factors within the urbanization and altitude affected the microbial communities. The various variables measured in each group did not showed a direct influence in the microbial communities, due to the lack of significant *P*-values. Excluding, the relative environmental humidity that in certain cases showed significant differences between the ranges of humidity. The metabolism of amphibians requires high quantities of water, when the levels of humidity changes can cause a level of stress in the organisms (Silva et al., 2012). And so several responses start to regulate stress, which can mediate the growth of gut microbiome and elucidate their activity. Intestinal cells can produce neuroendocrine hormones that directly affect the microbial communities (Karl et al., 2018).

The most abundant groups of bacteria in the intestinal microbiome of *P. unistrigatus* were Firmicutes (mostly *Clostridiales*), Proteobacteria and Bacteroidetes. Different studies around the globe have shown the same dominance of this groups in the

intestinal microbiome of other species of anurans. However, looking closer the bacterial composition patterns were specific to each species (Vences et al., 2016; Weng, et al., 2016). A question for further investigation remains open: which is the role of each group inside the intestinal microbiome. For example, Proteobacteria which was one of the most abundant groups throughout all the samples, could help with the metabolism of amino acid substrates even in low concentrations (Beebee & Wong, 1992). On the other hand, predictions by dynamic interaction modeling between Firmicutes and Proteobacteria has suggested interactions among this microorganisms, but the complexity of this microbiome has made it difficult to have experimental data to examine this relationships (Weng et al., 2017).

The dominance of Clostridia in the low and rural groups suggest there are anaerobic conditions in the gastrointestinal system of *P. unistrigatus* (and could be one of the major differences with the skin microbiome) (Vences et al., 2016). Still this group is also related to inflammatory responses and reducing of the microbiome diversity in the human intestine (Karl et al., 2018). In addition changes like this in the organization of the microbiome could lead to alterations to the immune system being the primary sensor for microbes and their metabolites (Weng et al., 2016). The development and function of the innate and adaptive immune system of toads is highly influenced by the colonization of different microorganisms (Weng et al., 2016). An increase in environmental stress can lead to a corruption of the intestinal microbiome which also make anurans susceptible to bacterial infections leading to septicemia and even death of the organisms (Fedewa, 2006).

Stress can lead to dysbiosis in the gastrointestinal system harming the host health (Karl et al., 2018). For example, in hibernating frogs the metabolism decreases, reducing also the functioning of the intestinal microbiome. Revealing also that this could change

the species interaction and functioning with the ecosystem (Weng et. al., 2016). Studies have shown that the presence of filamentous bacteria can prevent the colonization of pathogenic bacteria in the gastrointestinal system of anurans. Then again the presence of filamentous bacteria in the intestine can vary due to diet and external variables (Klaseen et al., 1993).

Amphibians have evolved an intimate relationship with their microbial communities (Boni & Battaglini, 1964). Gut microorganisms can reflect an evolutionary selection driven by the external environment (Huang et al., 2018). The structure of microbial communities differ between elevations. High altitude can be an environmental stressor for the functioning of the gastrointestinal system. It can lead to the loss of appetite, nausea, abdominal pain, among others in humans. Although intestinal epithelial cells work normally under a gradient of oxygen, the lower proportion of oxygen has changed the composition of the intestinal microbiome (Karl et al., 2018). This suggests that the environmental conditions at high and low location does contribute with the composition of the gut microbiome. Different studies showed that there were relationships of the diversity and richness of bacteria with altitude patterns, moreover in Andean regions there were not previous reports in soil bacteria influence by it (Medina et al., 2017). Also a study of the human gut microbiome composition through an altitude range has shown structural differences among the groups. Where Firmicutes were more abundant in the group with the higher altitude, and Bacteroidetes was more abundant in the group of lower altitude (Li & Zhao, 2015), which is inverse to the results in this research where Firmicutes were more abundant in the lower groups.

Although there is no a consensus on what a ‘healthy’ microbiome looks like, there is a level of agreement on the characteristics where the composition is favorable for success of a certain species (Karl et al., 2018). The understanding of how this micro-

ecosystems work can generate a new focus of study of the ecology of amphibians around the world and so, the understanding of how the communities of this species in Ecuador could be adapting to the anthropogenic impacts in their ecological niches. The response and adaptation of the intestinal microbiome over environmental stressors can be promoting or degrading the health of the host. Is that so that the intestinal microbiome could be a factor that helps the host to outcome short and long term changes in their environment (Karl et al., 2018). Being this one of the first researches of frogs' intestinal microbiome in Ecuador, further investigation is needed to truly understand the dynamics inside and between the microbiome, the host and the environment where this species develops.

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Apendix

Figures

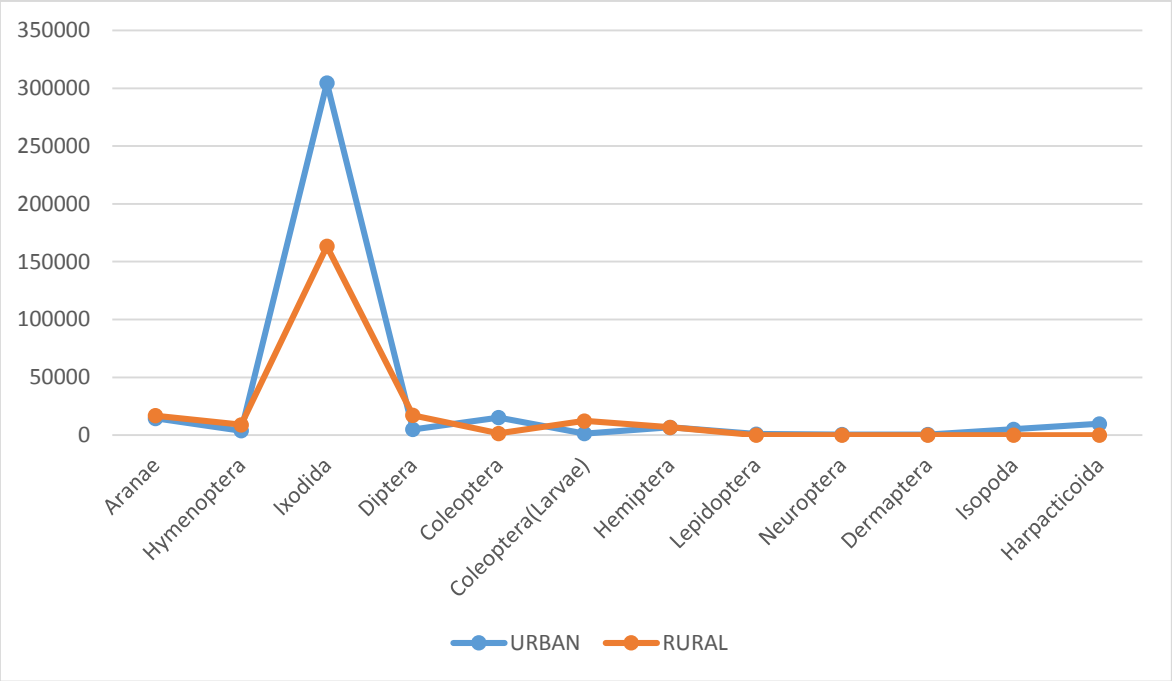

Figure 1: Differences in Index of Relative Importance of frog's preys between urban and rural habitats

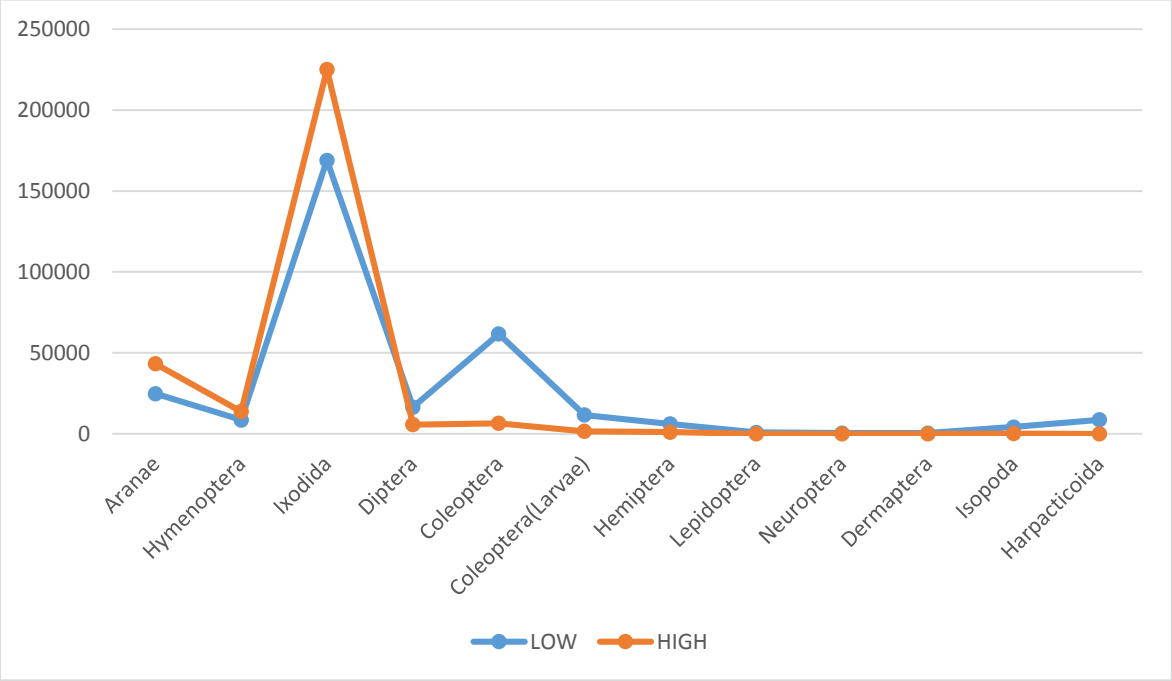

Figure 2: Differences in Index of Relative Importance of frog's preys between low and high habitats

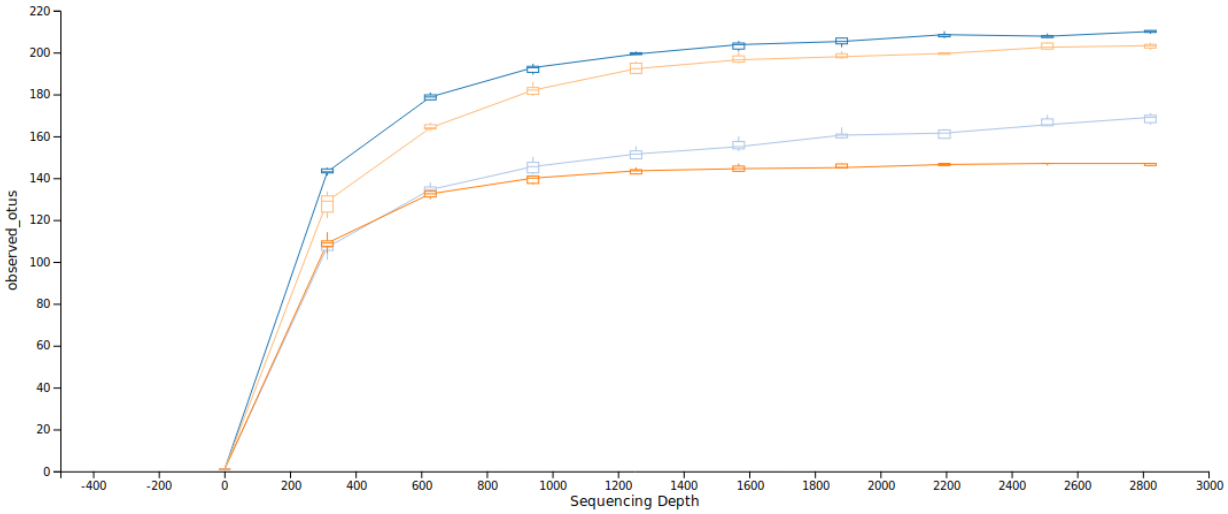

Figure 3: Rarefaction curves of Andean frogs based on Ilumina MiSeq sequencing. Horizontal axis number of sequenced data and vertical axis the observed number of the operational taxonomic units.

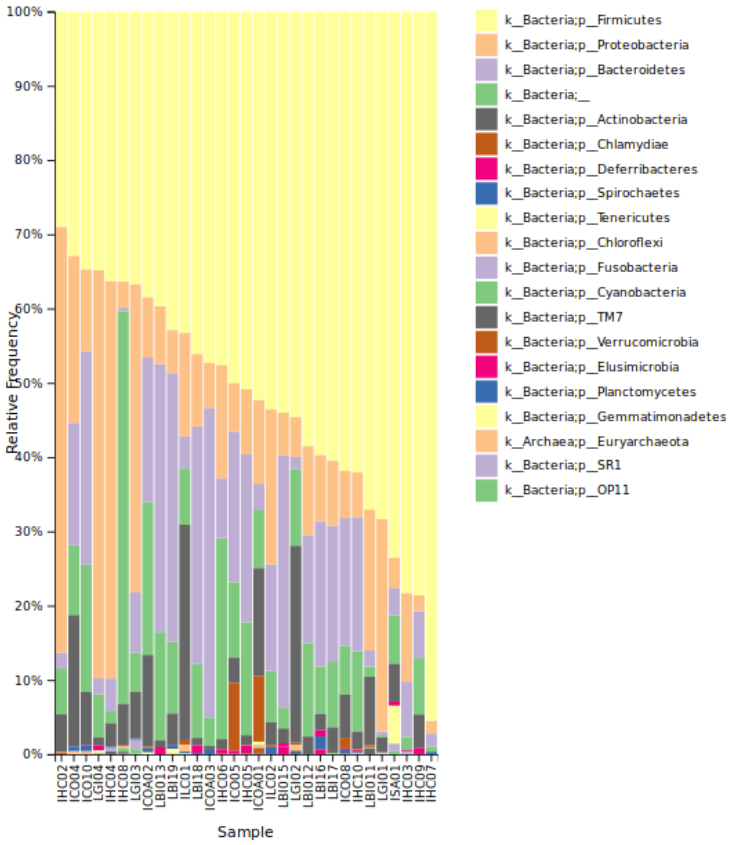

Figure 4: Relative frequency of microbial taxa based on Ilumina MiSeq.

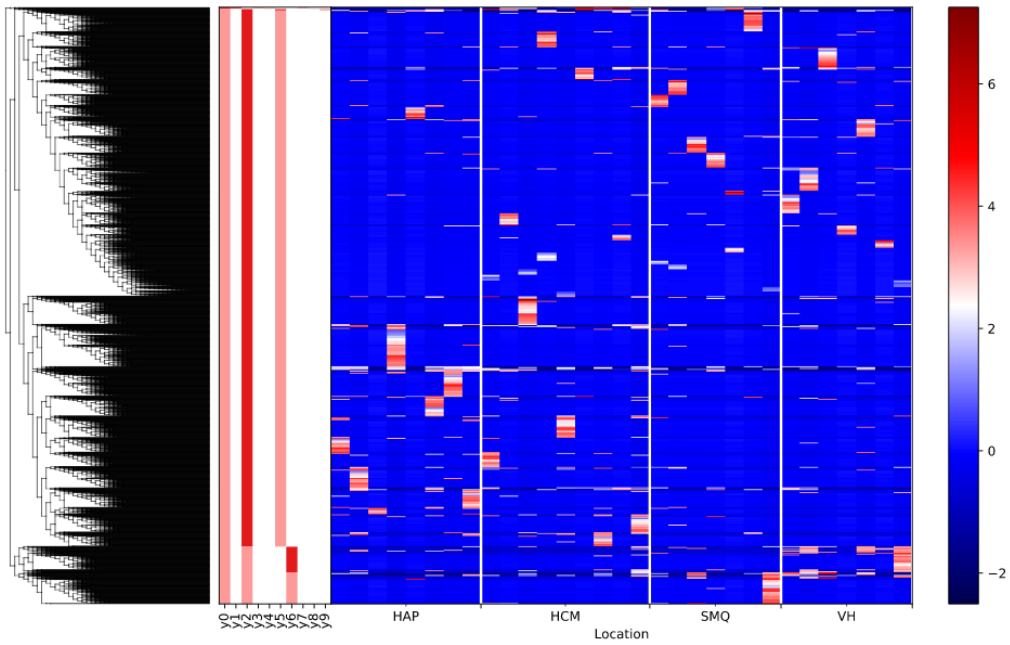

Figure 5: Heatmap of the relative abundance of the OTUs considered members across all sites. Each column represents a single frog sample.

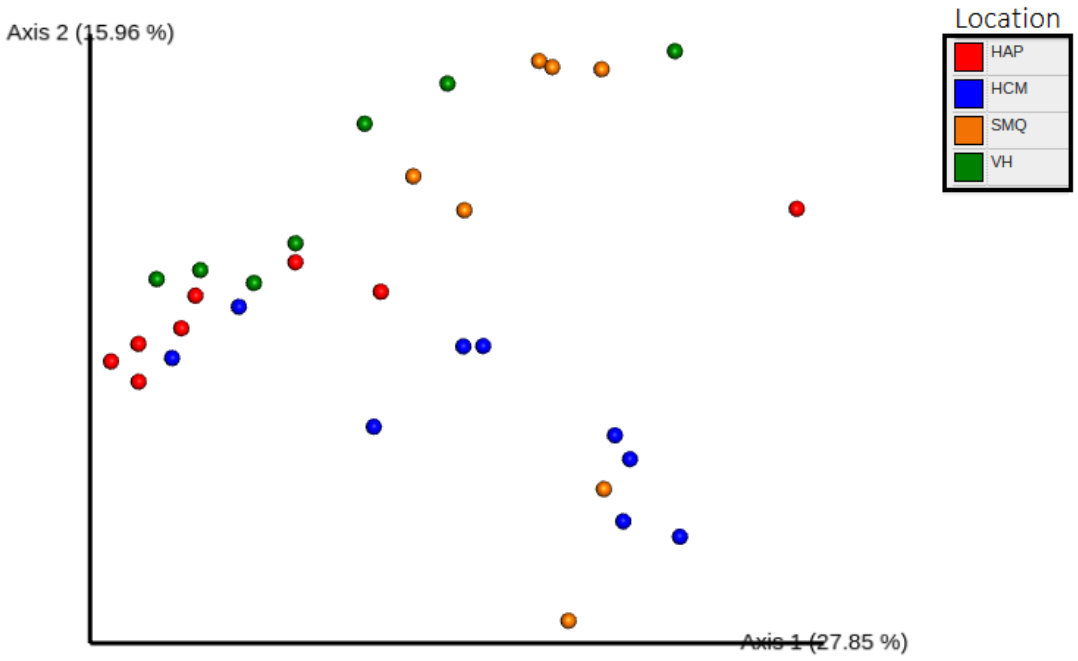

Figure 6: Beta diversity of intestinal microbial communities among the four sites based of the weighted UniFrac distance.

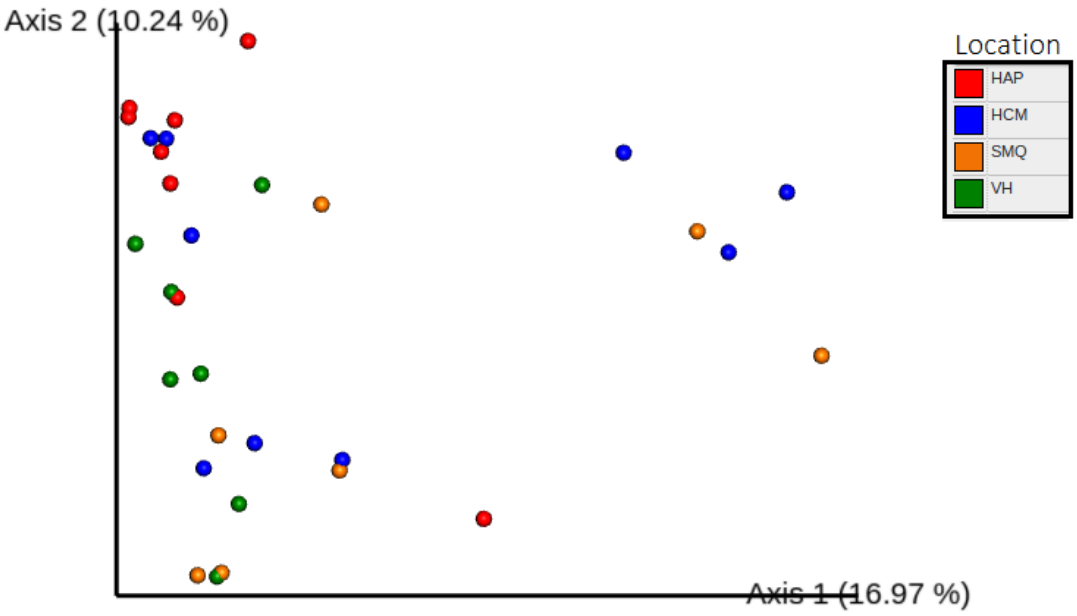

Figure 7: Beta diversity of intestinal microbial communities among the four sites based of the unweighted UniFrac distance.

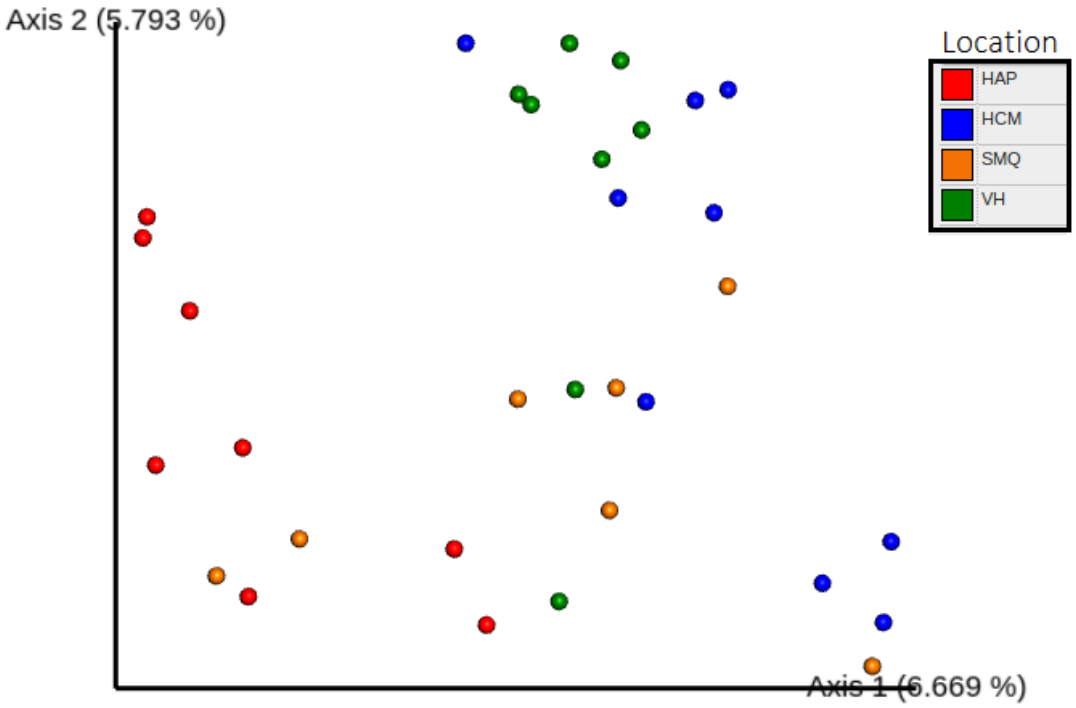

Figure 8: Beta diversity of intestinal microbial communities among the four elevations based on the Jaccard Scores

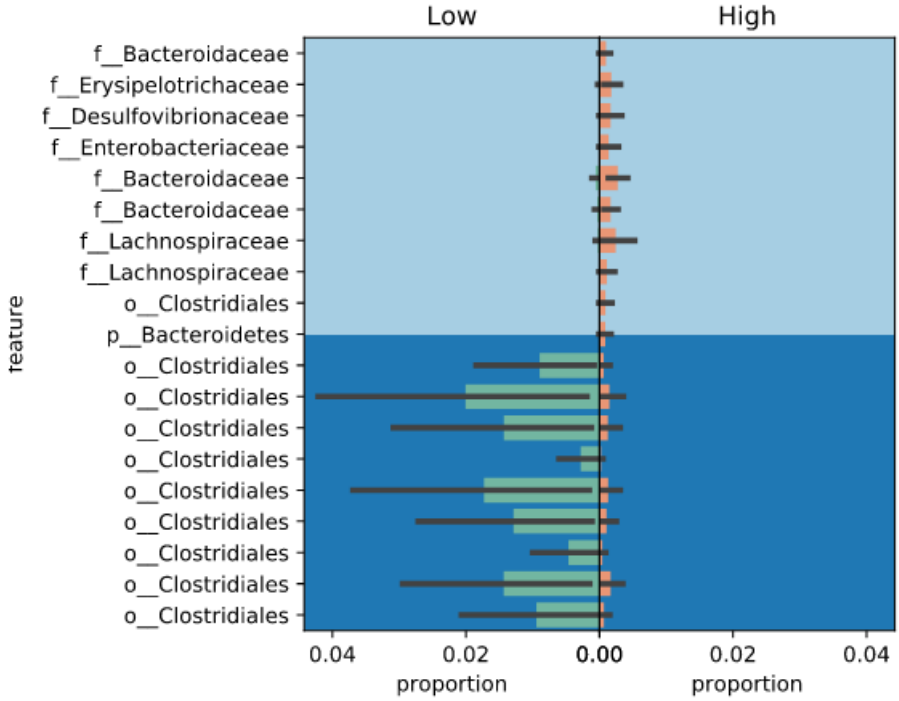

Figure 9: Proportion Plot of differential abundance analysis using balances in gneiss according to the elevation.

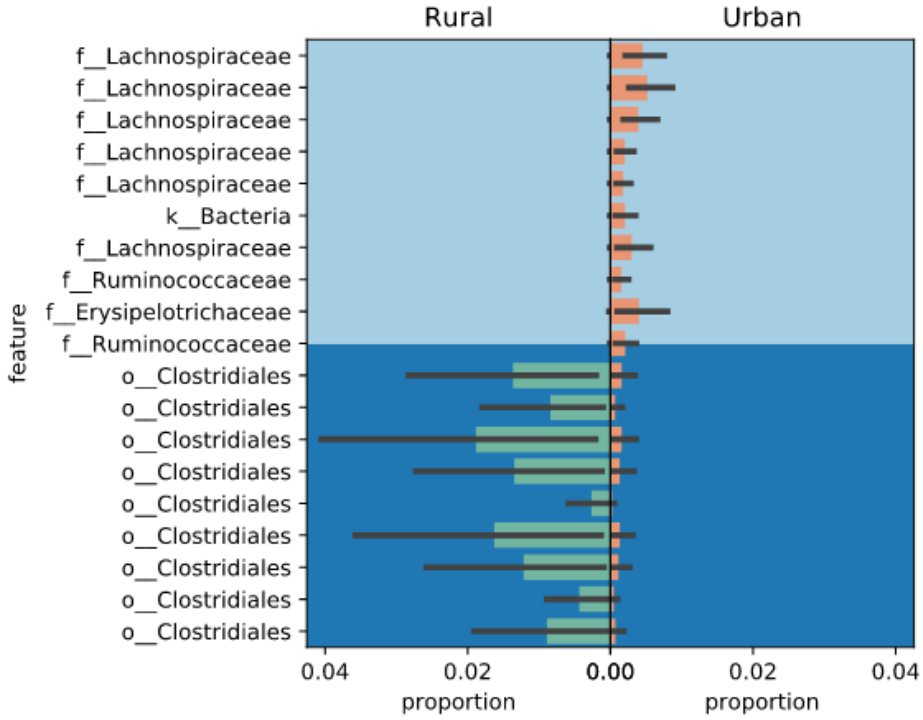

Figure 10: Proportion Plot of differential abundance analysis using balances in gneiss according to the urbanization.

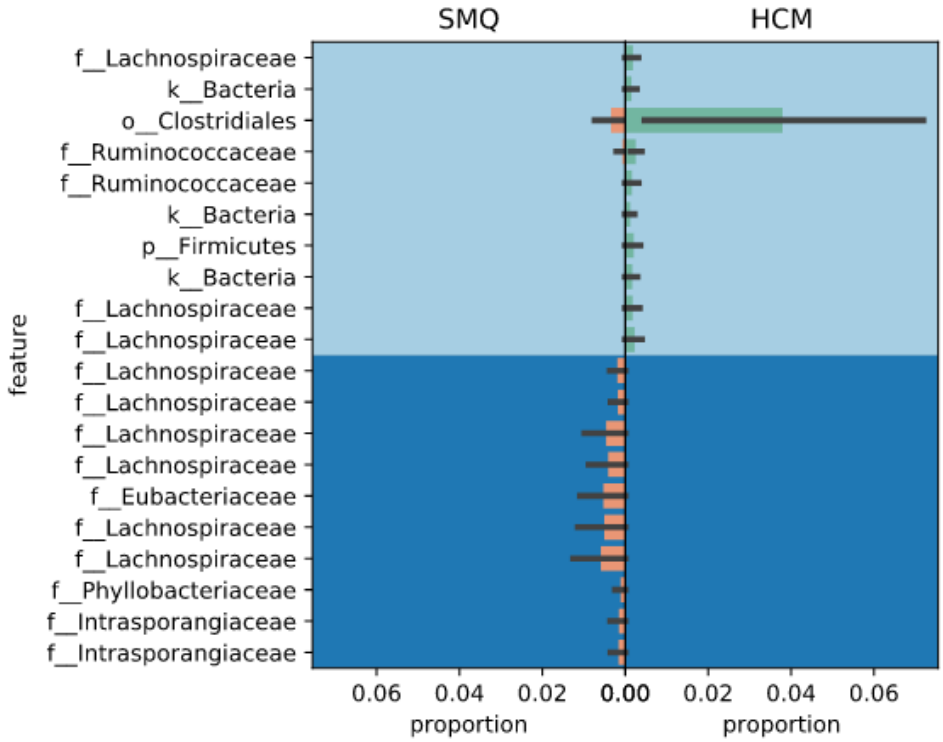

Figure 11: Proportion Plot of differential abundance analysis using balances in gneiss comparing two locations.

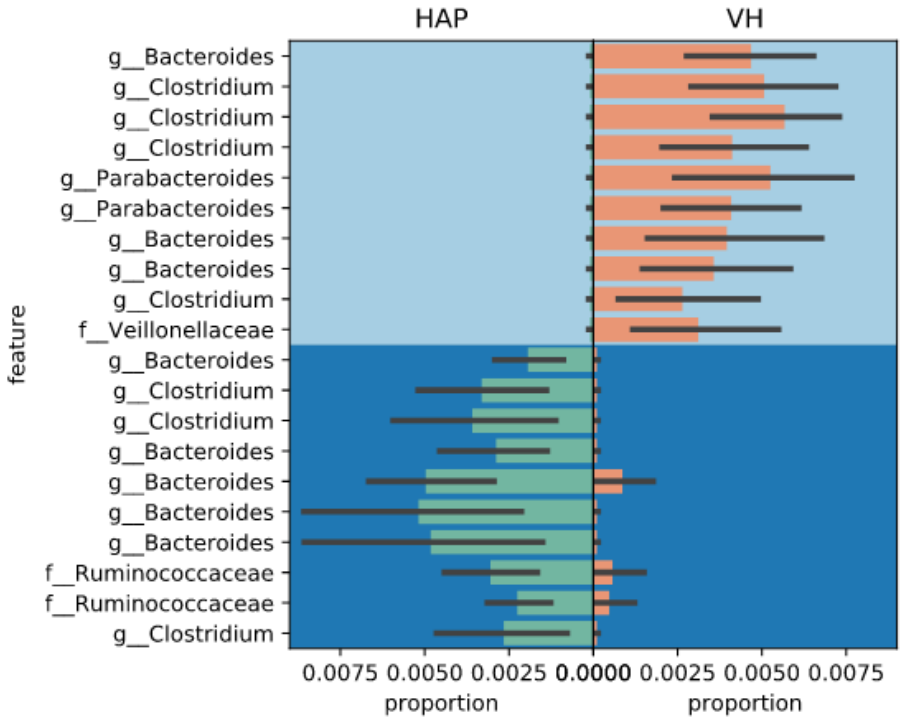

Figure 12: Proportion Plot of differential abundance analysis using balances in gneiss comparing two locations.

Figure 13: Map of each location in Quito.

Tables

Table 1: Index of relative Importance of the Stomach contents of *Pristimantis unistrigatus* in the four sites of collection

Prey Category	Urban	Rural	Low	High
Aranae	14520.5842	16878.1078	24765.976	43237.455
Hymenoptera	3893.34041	8955.25566	8533.83171	13770.7845
Ixodida	304584.05	163401.511	168990.935	225047.885
Diptera	5014.35804	17114.2385	16518.1107	5657.39717
Coleoptera	15183.5996	1487.6778	61682.7167	6530.21288
Coleoptera(Larvae)	1393.853	12346.2481	11608.0594	1509.25333
Hemiptera	6719.20561	6813.13594	6135.40718	1026.10658
Lepidoptera	976.170488	0	843.168724	0
Neuroptera	541.376399	0	475.667482	0
Dermaptera	519.552963	0	456.41151	0
Isopoda	5114.08447	0	4188.52367	111.870196
Harpacticoida	9833.61898	0	8674.70505	0

Table 2: T-test of stomach contents of *P. unistrigatus* in the four sites of collections

	Urban vs. Rural	Low vs. High
Number of Prey Item	0.896	0.400
Relative abundance	1.000	1.000
Volume of Prey (mm^3)	0.682	0.955

Table 3: Simpson's index and Coefficient symmetry of overlapping in the four sites of collections

	Simpson's Index	C. S. of Overlapping
High	0.751249519	
Low	0.803688281	
High vs. Low		0.951151658
Rural	0.729258559	
Urban	0.818359375	
Urban vs. Rural		0.86388799

Table 4: *P*-Values of Alpha diversity analysis (Evenness and Faith) between the four sites of collection

Location	Evenness	Faith
HAP vs. HCM	0.012	0.500
HAP vs. SMQ	0.015	0.132
HAP vs. VH	0.643	0.643
HCM vs. SMQ	0.100	0.368
HCM vs. VH	0.022	0.873
SMQ vs. VH	0.084	0.337

Table 5: *P*-Values of Beta diversity analysis based on weighted and unweighted UniFrac distances between the four sites of collection

Location	Unweighted	Weighted
HAP vs. HCM	0.044	0.036
HAP vs. SMQ	0.012	0.010
HAP vs. VH	0.006	0.173
HCM vs. SMQ	0.619	0.140
HCM vs. VH	0.004	0.008
SMQ vs. VH	0.012	0.006

Table 6: *P*-Values of Beta diversity analysis based on weighted UniFrac distances between the sites of collection comparing the ranges of soil temperature

Group 1	Group 2	VH vs. SMQ	VH vs. HCM
12-13°C	13-14°C	0.098	-
	14-15°C	0.262	-
	15-16°C	0.274	-
	16-17°C	1.000	-
13-14°C	14-15°C	0.521	0.032
	15-16°C	0.426	0.134
	16-17°C	0.246	0.058
14-15°C	15-16°C	0.047	0.030
	16-17°C	0.428	0.067
15-16°C	16-17°C	1.000	0.681

Table 7: *P*-Values of Beta diversity analysis based on unweighted UniFrac distances between the sites of collection comparing the ranges of soil temperature

Group 1	Group 2	VH vs. HCM	SMQ vs. HCM
12-13°C	14-15°C	-	0.663
	15-16°C	-	0.036
	16-17°C	-	0.595
13-14°C	14-15°C	0.064	-
	15-16°C	0.104	-
	16-17°C	0.089	-
14-15°C	15-16°C	0.029	0.040
	16-17°C	0.385	0.391
15-16°C	16-17°C	0.461	0.386

Table 8: *P*-Values of Beta diversity analysis based on weighted UniFrac distances between the sites of collection comparing the ranges of environmental relative humidity

Group 1	Group 2	SMQ vs. HAP	HAP vs. HCM
40-60%	80-90%	0.307	-
	90-93%	0.818	-
	94-97%	0.068	-
	98-100%	0.085	-
80-90%	90-93%	0.619	-
	94-97%	0.066	-
	98-100%	0.098	-
90-93%	94-97%	0.025	0.205
	98-100%	0.029	0.019
94-97%	98-100%	0.112	0.702

Table 9: *P*-Values of Beta diversity analysis based on unweighted UniFrac distances between the sites of collection comparing the ranges of environmental relative humidity

Group 1	Group 2	SMQ vs. HAP
40-60%	80-90%	0.0331
	90-93%	0.674
	94-97%	0.066
	98-100%	0.109
80-90%	90-93%	0.729
	94-97%	0.060
	98-100%	0.117
90-93%	94-97%	0.037
	98-100%	0.020
94-97%	98-100%	0.054