



Fecal bacterial communities of wild black capuchin monkeys (*Sapajus nigritus*) from the Atlantic Forest biome in Southern Brazil are divergent from those of other non-human primates

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ABSTRACT

Gut microbiota are influenced by factors such as diet, habitat, and social contact, which directly affect the host's health. Studies related to gut microbiota in non-human primates are increasing worldwide. However, little remains known about the gut bacterial composition in wild Brazilian monkeys. Therefore, we studied the fecal microbiota composition of wild black capuchin monkey (*Sapajus nigritus*) (n=10) populations from two different Atlantic Forest biome fragments (five individuals per fragment) in south Brazil. The bacterial community was identified via the high-throughput sequencing and partial amplification of the *16S rRNA* gene (V4 region) using an Ion Personal Genome Machine (PGMTM) System. In contrast to other studies involving monkey microbiota, which have generally reported the phyla Firmicutes and Bacteroidetes as predominant, black capuchin monkeys showed a high relative abundance of Proteobacteria (\bar{x} = 80.54%), followed by Firmicutes (\bar{x} = 12.14%), Actinobacteria (\bar{x} = 4.60%), and Bacteroidetes (\bar{x} = 1.31%). This observed particularity may have been influenced by anthropogenic actions related to the wild habitat and/or diet specific to the Brazilian biome's characteristics and/or monkey foraging behavior. Comparisons of species richness (Chao1) and diversity indices (Simpson and InvSimpson) showed no significant differences between the two groups of monkeys. Interestingly, PICRUST2 analysis revealed that metabolic pathways present in the bacterial communities were associated with xenobiotic biodegradation and the biosynthesis of secondary metabolites, which may suggest positive effects on monkey health and conservation in this anthropogenic habitat. Infectious disease-associated microorganisms were also observed in the samples. The present study provides information about the bacterial population and metabolic functions present in fecal microbiota, which may contribute to a better understanding of the ecology and biology of black capuchin monkeys living in forest fragments within the Atlantic Forest biome in southern Brazil. Additionally, the present study demonstrates that the fecal bacterial communities of wild black capuchin monkeys in this area are divergent from those of other wild non-human primates.

Abbreviations: SSC, São Sebastião do Caí; SCS, Santa Cruz do Sul; PGMTM, Personal Genome Machine; FastQC, Fast Quality Control; MultiQC, Multi Quality Control; FROGS, Find Rapidly OTUs with Galaxy Solution; OTUs, Operational Taxonomic Units; SSU, Small Subunit rRNA gene; PICRUST2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved State; KEGG, Kyoto Encyclopedia of Genes and Genomes; HTS, high-throughput sequencing.

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Introduction

Primates play an important role in maintaining well-functioning forest ecosystems since they serve as key ecological agents in the environment. They play important roles in the livelihoods, cultures, and religions of many societies, contribute to plant pollination and seed dispersal, and also serve as indicator species since the health of their populations reflects the general health of an ecosystem (Marshall and Wich, 2016; Estrada et al., 2017). However, primate populations have become threatened in recent years due to anthropogenic factors such as habitat loss, hunting and trapping, logging and wood harvesting, pollution, and climate change (Estrada et al., 2017).

Of the world's 504 non-human primate species, two-thirds of them occur in Brazil, Madagascar, Indonesia, and the Democratic Republic of the Congo (Estrada et al., 2017). According to Quintela et al. (2020), there are 129 primate species in Brazil, which belong to five families: Callitrichidae, Pitheciidae, Cebidae, Atelidae, and Aotidae. The genera *Cebus* (gracile capuchin monkeys, without head tufts) and *Sapajus* (robust capuchin monkeys, with head tufts) are members of the subfamily Cebinae and endemic to Brazilian biomes. Notably, they occur in the driest (e.g., the Cerrados and Caatingas) and most humid (e.g., the Amazon and Atlantic Forest) biomes (Martins-Junior et al., 2018). Currently, eight species of *Sapajus* are recognized in Brazilian biomes: *Sapajus macrocephalus* and *Sapajus apella* (Amazon biome); *Sapajus libidinosus* and *Sapajus cay* (Caatinga and Cerrado biomes); *Sapajus xanthosternus*, *Sapajus robustus*, *Sapajus nigritus cucullatus*, *Sapajus nigritus nigritus*, and *Sapajus flavius* (Atlantic Forest biome) (Martins-Junior et al., 2018; Quintela et al., 2020).

Robust capuchins (*Sapajus* spp.) are considered the most intelligent Neotropical primates due to their flexible social and feeding behaviors (Ludwig et al., 2005; Rímoli et al., 2008; Izar et al., 2012; Lowry et al., 2013; Aguiar et al., 2014; Falótico et al., 2018; La Salles et al., 2018; Back et al., 2019). They are medium-sized Neotropical primates (adults measure 30–56 cm and weigh 3.9–2.5 kg) that have a life expectancy of approximately 44 years in captivity and 25 years in nature (Cardoso, et al., 2021). They live in groups of approximately 10 to 30 individuals, with females tending to be philopatric and males tending to migrate before reaching sexual maturity (Izar et al., 2012; Cardoso, et al., 2021). The social structure of robust capuchins involves hierarchies of dominance among males and females (Cardoso, et al., 2021). They are true habitat generalists with an incredible dietary breadth compared to other Neotropical primates (Aguiar et al., 2014; Cardoso, et al., 2021). Their diet includes a wide variety of fruit and insects, which form the bulk of their diets. Moreover, their robust jaw morphology and behavioral adaptations for tool use and manipulative and extractive foraging allow for the exploitation of encased and hidden foods unavailable to most other non-human animals. The ability to obtain food using tools allows robust capuchins to diversify their diet and helps them easily adapt to a diverse range of environments (Aguiar et al., 2014). In fragmented patches of the Atlantic Forest biome, robust capuchins adjust their foraging patterns by exploring new feeding resources (i.e., artificial or exotic foods). Simultaneously, they must cope with stressful situations such as hunting, dog predation, electrocution, exposure to anthropogenic pollutants, intoxication, human conflict, and exposure to pathogens from humans and domesticated animals (Aguiar et al., 2014; Estrada et al., 2017). These interactions may have serious consequences for robust capuchins since bringing them into contact with a wide diversity of microorganisms might affect their health and conservation.

The resilience and persistence of some primate species to environmental changes depend on their phenotypic plasticity and/or microevolution, as well as the interactions with their gut microbiota (Barelli et al., 2020). These interactions can affect host health and behavior, appear to shape host fitness in a variety of contexts, and represent key factors in existing models of human and primate ecology and evolution (Clayton et al., 2018). Notably, the knowledge of primate microbiota has been increasing in recent years (Firmann et al., 2019; Hale et al., 2019),

while factors such as diet (Greene et al., 2019b; Orkin et al., 2019^{a,b}), birth form (Rendina et al., 2019), habitat (Grieneisen et al., 2019), contact with humans (Grant et al., 2019), phylogeny (Gogarten et al., 2018; Amato et al., 2019), stress responses (Vlčková et al., 2018), age (Mitchell et al., 2018) and climate change (Sun et al., 2021) have been shown to influence gut microbiota composition and diversity.

An increasing number of studies have been conducted to investigate the gut microbiota in non-human primates, many of which were conducted on captive animals from China and the United States (Koo et al., 2019; Dettmer et al., 2019; Adriansjach et al., 2020; Wu et al., 2020; Ni et al., 2021) (Supplementary Table 1). To date, few studies have evaluated the gut microbiota in non-human primates from Central and South America (Mallott and Amato, 2018; Mallott et al., 2018; Orkin et al., 2019^a; Orkin et al., 2019^b; Garber et al., 2019; Malukiewicz et al., 2019; Compo et al., 2021; Janiak et al., 2021; Quiroga-González et al., 2021). For the subfamily Cebidae, only two studies have been conducted on wild white-faced capuchins (*Cebus capucinus imitator*) from Costa Rica (Orkin et al., 2019^a; Orkin et al., 2019^b). However, no studies have described the gut microbiota of robust capuchins (*Sapajus* spp.) and a few have used culture-dependent methods (Grassotti et al., 2018; Sobreira et al., 2019; Ehlers et al., 2020; Zaniolo et al., 2020).

After five centuries of human expansion, the Atlantic Forest of Brazil faces a higher extinction rate than other Brazilian biomes. Most Atlantic Forest landscapes are composed of small forest fragments surrounded by open habitat matrices such as pastures and agricultural fields (Joly et al., 2014). Habitat degradation and exploitation are the greatest threats to biodiversity and pose serious problems for both wildlife and humans. According to the IUCN (2015), the black capuchin monkey population is relatively large (~10,000) present in seven Brazilian states (i.e., Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul). However, since this population is experiencing ongoing decline due to habitat fragmentation, urban sprawl, road and energy network development, hunting, and hybridization, the black capuchin monkey is listed as near threatened (IUCN, 2015). Although Brazil is considered a hotspot for primate conservation, studies of the wild black capuchin monkey microbiome are nonexistent. Thus, studies that evaluate the gut microbiota in wild primates in the anthropogenic environment are needed to further understand the imminent threats faced by these animals.

The evaluation of gut microbial diversity and composition helps to provide a greater understanding of the resilience of wild primates and facilitates captive population management. In this context, the present study aimed to characterize and predict the general metabolic profiles of fecal bacterial communities of two populations of wild black capuchin monkeys (*S. nigritus*) living in fragments of the Atlantic Forest biome in Southern Brazil. The present study increases our understanding of the fecal microbiota in wild non-primates and suggests that the resilience and adaptation of wild black capuchin monkeys to habitat degradation and fragmentation in the Atlantic Forest biome may be associated with their bacterial community. Besides, our study compared the fecal bacterial communities of wild black capuchin monkeys with those encountered in other non-human primates.

Materials and methods

Study area

The study area comprises two forest fragments of the Atlantic Forest biome in Rio Grande do Sul, Brazil. Notably, these forest fragments share the same phytophysiognomy (deciduous seasonal forest). This region typically features ombrophilous forest biomes with both wet and dry seasons. The climate of this region is subtropical and humid all year, with an average temperature exceeding 22 °C during the hottest month. Despite being hot and humid during most of the year, the climate preserves a cold character, for an appreciable period. This imposes restrictions on the proliferation and development of a large number of

typically tropical species. This type of forest has a high level of biodiversity and hosts one of the highest concentrations of endemic species in South America. At present, only 7.5% of the original Atlantic Forest is remaining, with a high degree of fragmentation in relation to the original vegetation cover (Socioeconomic Atlas of Rio Grande do Sul, 2019; Morellato and Haddad, 2000).

The first population of wild black capuchin monkeys lives in a forest fragment located at São Sebastião do Caí (SSC) city (29° 35' 13" S; 51° 22' 17" W) and is composed of 20–30 individuals. The second population lives in a forest fragment located within the Parque Municipal da Gruta dos Índios in Santa Cruz do Sul (SCS) city (29° 43' 03" S; 52° 25' 33" W). This population consists of 30–40 individuals (Supplementary file).

The wild black capuchin monkeys inhabiting these forest fragments eat plants and insects. Additionally, since they live near urban areas, they also have access to unintentional (e.g., through garbage) or intentional feeding by humans (e.g., fruits, vegetables, and industrialized foods such as snacks, sweets, fried foods, and more) (Wenzel and Quadro, 2012).

Sample collection

Ten rectal swabs were collected from two groups of wild black capuchin monkeys using a sterile swab introduced 3–5 cm into the rectum and rotated 360° (Table 1). Swabs were immediately placed into the Stuart transport medium (Kasvi, Paraná, Brazil) and kept at 4°C until DNA extraction.

The animals were captured using Tomahawk-type cage traps. Ketamine (100 mg/mL) and xylazine (20 mg/mL) were administered intramuscularly for wild animal immobilization (Miranda et al., 2011). Rectal swabs were collected by trained veterinarians. Physiological parameters such as rectal temperature, heart rate, and respiratory rate were recorded and used to indirectly measure the animals' welfare status. An ANOVA was used to assess the statistical difference between the weights of the animals. All animals were clinically healthy and classified according to gender and age group (Fragaszy et al., 2004). After sample collection, the animals were returned to their habitats.

The animals were captured and manipulated using conventional methods according to the protocol for sample collection described by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, 2012). The capture and manipulation of monkeys for sample collection were authorized by the Brazilian Institute of Environment and Renewable

Table 1
Details of wild black capuchin monkeys (*Sapajus nigritus*) analyzed in this study.

Forest fragment	Sample (ID)	Gender	Age	Weight (kg)	Appearance overall	Collection date
SSC	BCM 8	F	A	2.620	Healthy*	23/08/2016
	BCM 9	F	Y	1.520	Healthy	23/08/2016
	BCM 12	F	A	2.075	Healthy	23/08/2016
	BCM 14	M	Y	1.560	Healthy	23/08/2016
	BCM 15	M	Y	2.200	Healthy	23/08/2016
SCS	BCM 23	M	A	3.755	Healthy	23/05/2017
	BCM 24	M	A	3.170	Healthy	23/05/2017
	BCM 26	F	A	2.270	Healthy	23/05/2017
	BCM 27	M	A	2.000	Healthy	23/05/2017
	BCM 28	M	A	2.200	Healthy	23/05/2017

SSC: São Sebastião do Caí; SCS: Santa Cruz do Sul; BCM: black capuchin monkey; M: male; F: female; A: adult; Y: young; *lactating.

Natural Resources (IBAMA), Brasília, Brazil, and the ICMBio. The protocol was approved by the Information Authorization System in Biodiversity (SISBIO) (number 56640). This project is registered in the National System for the Management of Genetic Heritage and Associated (SISGEN) (numbers A2CC618 and A720680).

DNA extraction

Total DNA from rectal swabs samples was extracted using MoBio's PowerSoil DNA extraction kit (ThermoFisher Scientific), according to the manufacturer's instructions. The DNA concentration was determined using the Qubit, and its quality was verified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR-amplification of bacterial 16S rRNA gene and sequencing

To characterize the bacterial community present into each fecal sample, the fragments of V4 region of the 16S rRNA gene were amplified using the primers 515F and 806R (Caporaso et al., 2011) and further sequenced using a PGM™ Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA). Multiple samples were PCR-amplified using barcoded primers linked with the Ion adapter "A" sequence and Ion adapter "P1" sequence to obtain a sequence of primer composed for A-barcode-806R and P1-515F adapter and primers.

PCR assays were performed with the Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA), in a volume of 25 µL containing 1 × High Fidelity PCR buffer, 2U of Taq Polymerase, 2 mM MgSO₄, 0.2 mM dNTP Mix, 25 µg of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA), 0.1 µM of each primer and approximately 50 ng of DNA template and ultrapure water to complete one volume. The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 56°C for 45 s, and 68°C for 1 min, and a final extension of 68°C for 10 min.

Samples were sequenced at the Federal University of Pampa (UNIPAMPA, São Gabriel, RS, Brazil). After purifying PCR amplicons using Agencount AMPure Beads (Beckman Coulter), library preparation with the Ion OneTouch™ 2 System fitted with the Ion PGM™ OT2 400 Kit Template (Thermo Fisher Scientific, Waltham, MA, USA) from an initial amount of 100 ng of PCR product. Since all samples were sequenced in a multiplexed PGM™ run, barcode sequences were used to identify each sample from the total sequencing output. Sequencing was conducted using a chip with Ion 316 chips, following the manufacturer's instructions. Sequences have been submitted and published to the EMBL database under accession number PRJEB35777. Despite the short read lengths (~290 bp), this targeted gene region provides sufficient resolution.

Bacterial community analysis

The raw data quality was evaluated with FastQC (Andrews, 2018) and a summary report was constructed with MultiQC (Ewels et al., 2016). Elimination of the adapters was done with Cutadapt v.2.3 (Martin, 2011), and the quality-filtered sequences were imported into the FROGS (Find Rapidly OTUs with Galaxy Solution) pipeline (Escudé et al., 2018) to obtain the Operational Taxonomic Units (OTUs). The sequences were filtered by length (250–300 bp) and then pooled into OTUs with SWARM (Mahé et al., 2015) with the distance parameter d = 3. Chimeras were removed with VSEARCH (Rognes et al., 2016) and OTUs were retained with at least 190 reads in the whole dataset (corresponding to 0.1%). These steps resulted in the retention of OTUs, which were affiliated with SILVA 132 SSU databases (Quast et al., 2013), delimited at 97% identity (Edgar, 2018).

The bacterial diversity analyses were performed in R Studio v. 4.0.3 using the phyloseq package (v1.30.0) (McMurdie and Holmes, 2013). The relative abundance of species present in the samples was plotted with the plot_composition function. The taxon diversity study (richness and evenness) within the samples was estimated with plot_richness and

plotted with boxplot function using Chao1, Simpson and Inverse Simpson indexes. The numeric values were estimated with estimate_richness and statistics were performed with ANOVA and linear model regression. A heatmap with the OTU abundances at family levels was plotted using the plot_heatmap function from the ggplot2 package v3.3.0 (Wickham et al., 2010) and DESeq2 was applied to visualize differentially abundant OTUs (Love et al., 2014). A hierarchical clustering based on Jaccard distance was plotted with the ward.D2 method and permanova tests was performed between the two regions. All codes are available as supplementary material and can be found on the online version of this article.

Functional predictions from amplicon sequences

A predictive functional profile of the fecal bacterial community was conducted using PICRUSt2 software (v2.3.0) (Douglas et al., 2020). Briefly, the OTU sequences were executed using the script picrust2_pipeline.py. This script run each of the 4 key steps: (1) sequence placement, (2) hidden-state prediction of genomes, (3) metagenome

prediction, (4) pathway-level predictions. The functions predicted were categorized using the mappings table from the KEGG database (Kanehisa et al., 2017). DESeq2 method was used to determine the statistical significance of the metabolic functions between SSC and SCS monkeys (Love et al., 2014). The codes used are available at github (https://github.com/Carolkothe/Brazilian_Monkey) and all associations that produced an adjusted p-value >0.05 (using Benjamini-Hochberg method) were considered insignificant.

Results

Fecal bacterial composition of wild black capuchin monkeys

A total of 162,125 high-quality reads were obtained from the fecal samples of wild black capuchin monkeys. The DNA sequences were grouped into 84 OTUs, while seven phyla presenting relative abundances greater than 0.1% were observed in the evaluated samples. Among them, Proteobacteria exhibited the highest relative abundance

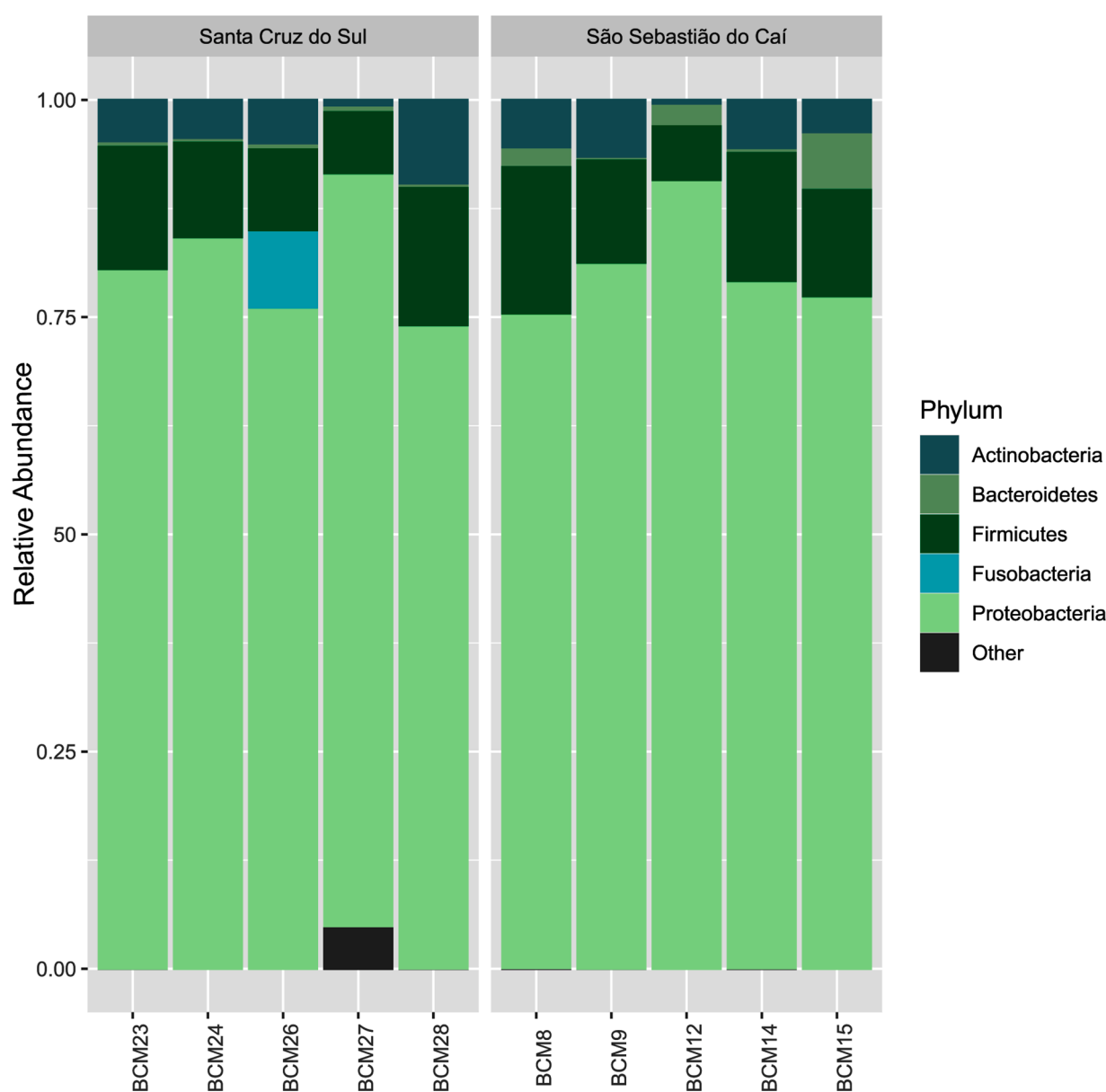


Fig. 1. Fecal bacterial composition of wild black capuchin monkeys (*Sapajus nigritus*). Taxonomic composition of the fecal microbiota found among 10 samples separated into two different locations (Santa Cruz do Sul and São Sebastião do Cai) fragments of Atlantic Forest biome in the Rio Grande do Sul State, Brazil was compared based on the relative abundance (reads per sample).

(\bar{x} = 80.54%), in the fecal microbiota of wild black capuchin monkeys, followed by Firmicutes (\bar{x} = 12.14%), Actinobacteria (\bar{x} = 4.60%), Bacteroidetes (\bar{x} = 1.31%), and other phyla (\bar{x} = 1.41%) (Fig. 1). The distribution of phyla in the fecal microbiota of wild black capuchin monkeys was similar between the two groups ($p > 0.05$ for all phyla) despite their geographic distance of 121 km (Fig. 1). In both groups, no significant differences with respect to the body weight were observed (Supplementary Fig. 1.)

A total of 142 families were detected in fecal samples; however, only

45 (\bar{x} = 34.10%) showed a relative abundance of $\geq 0.1\%$. In general, their distribution in the fecal microbiota of all wild black capuchin monkeys was very similar among the samples. The dominant families in the evaluated samples were Enterobacteriaceae (\bar{x} = 21.58%), Morganellaceae (\bar{x} = 20.89%), Burkholderiaceae (\bar{x} = 18.42%), and Streptococcaceae (\bar{x} = 4.38%) (Supplementary Fig. 2). Fecal bacterial composition results were complemented with heatmap analysis (Fig. 2).

Alpha diversity metrics (i.e., the Chao1, Simpson, and InvSimpson indices) did not exhibit any identifiable changes ($p > 0.05$) in bacterial

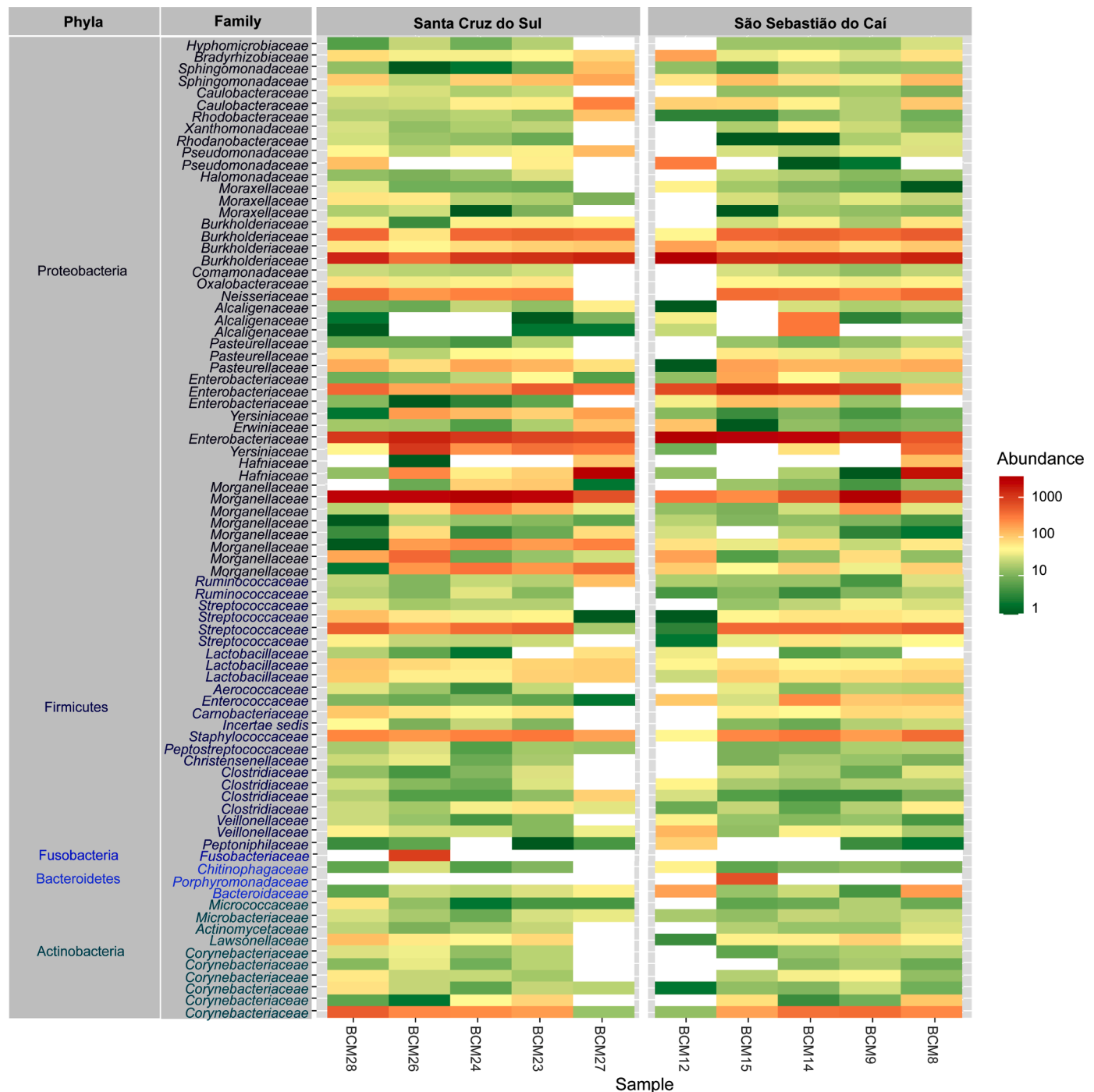


Fig. 2. Heatmap showing fecal bacterial phyla and family relative abundances among wild black capuchin monkeys from two different locations (Santa Cruz do Sul and São Sebastião do Cai) from fragments of Atlantic Forest biome in the Rio Grande do Sul State, Brazil. Each column represents an individual monkey, with the header colored according to the local sample. Color within the heatmap represents intensity of OTU relative abundances along a color scale gradient provided, where larger values represent a higher relative abundance. BCM: black capuchin monkey. Strong red indicates high positive correlation and strong green is high negative correlation.

community structure grouping from the two populations of wild black capuchin monkeys within the Atlantic Forest biome in southern Brazil (Fig. 3A; Supplementary Table 3).

Principal coordinate analysis (PCoA) plots using a binary Jaccard distance of fecal bacterial communities were used to generate the beta diversity distance matrices and calculate the degree of differentiation among samples. The mean distances between all groups were calculated and no statistically significant differences were observed between the quantitative and qualitative composition of the fecal microbiota according to the groups ($p > 0.05$) (Fig. 3B–C).

Fecal microbial functional profile prediction of wild black capuchin monkeys

The metabolic functions provided by PICRUST2 using the 16S rRNA gene amplicon resulted in predicted proteins classified as KEGG orthologs (KOs), with a total of 254 KOs across all hosts. Moreover, the results

of the statistical test nbinomTest (DESeq2) (Anders and Huber, 2010) showed no difference in predicted function between monkeys from two forest fragments (SSC and SCS) in the KEGG pathways based on their fecal microbiomes.

Twenty pathways were observed into a two-level category for KEGG pathways, including membrane transport, amino acid metabolism, carbohydrate metabolism, energy metabolism, replication and repair systems, cofactor and vitamin metabolism, nucleotide metabolism, xenobiotic biodegradation metabolism, lipid metabolism, the metabolism of other amino acids, polypeptide and terpenoid metabolism, the biosynthesis of other secondary metabolites, and others (Supplementary Table 2). A network-wide analysis of co-occurrence dynamics between bacterial taxonomy (Fig. 4A) in the fecal samples of wild black capuchin monkeys and predicted metabolic pathways demonstrated a relationship between Proteobacteria, membrane transport, and poorly characterized metabolites.

Forty-two pathways were identified into a three-level category for

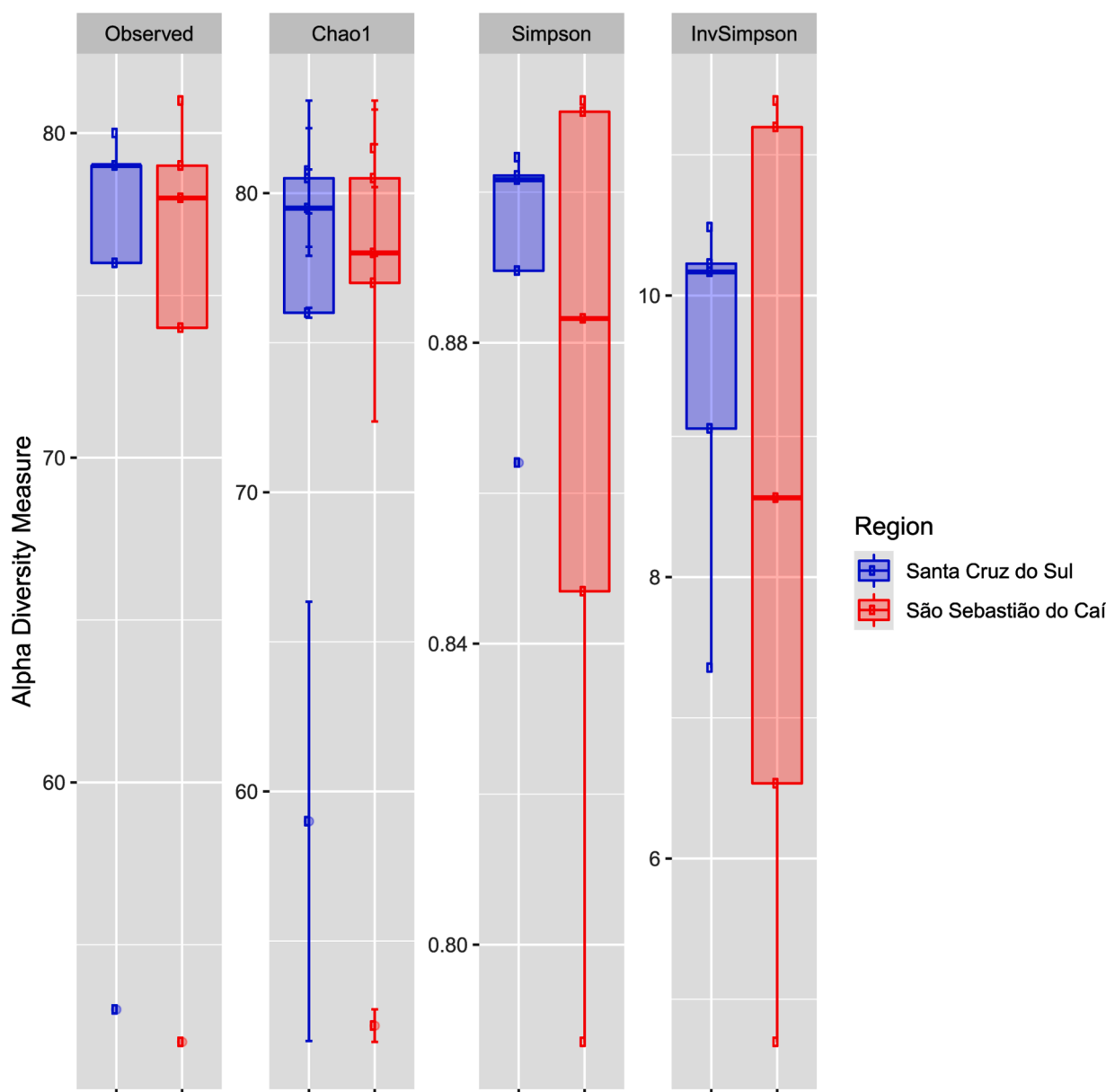


Fig. 3. Alpha- and beta-diversity analysis comparing monkeys fecal bacterial community. (A) Alpha-diversity analysis based on Chao1, Simpson and InvSimpson diversity. (B) Beta-diversity based on MDS+Jaccard distance matrix. (C) Hierarchical clustering of the monkey FMB using Jaccard distance demonstrates that no significant differences were observed among the samples. BCM: black capuchin monkey. Blue: Samples from Santa Cruz do Sul. Red: Samples from São Sebastião do Caí.

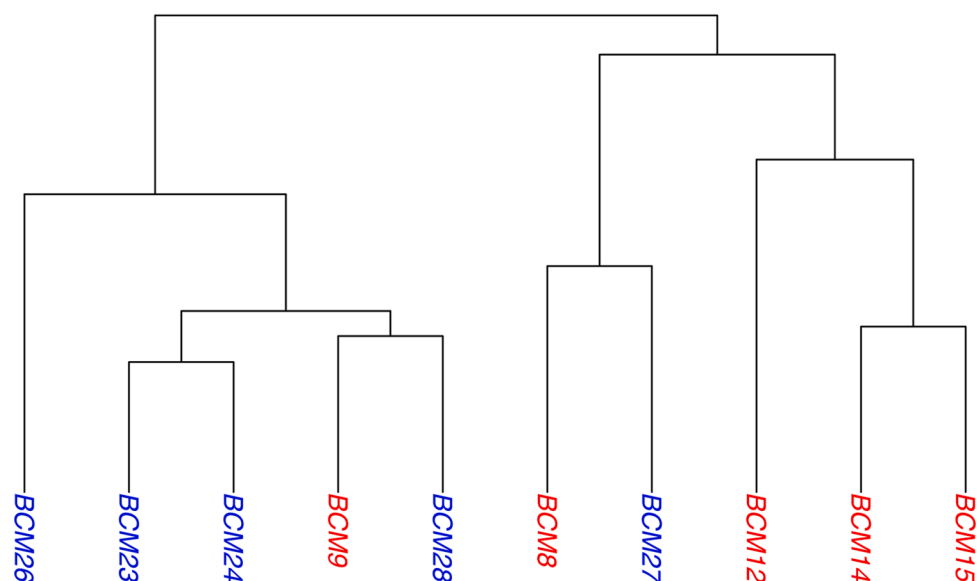
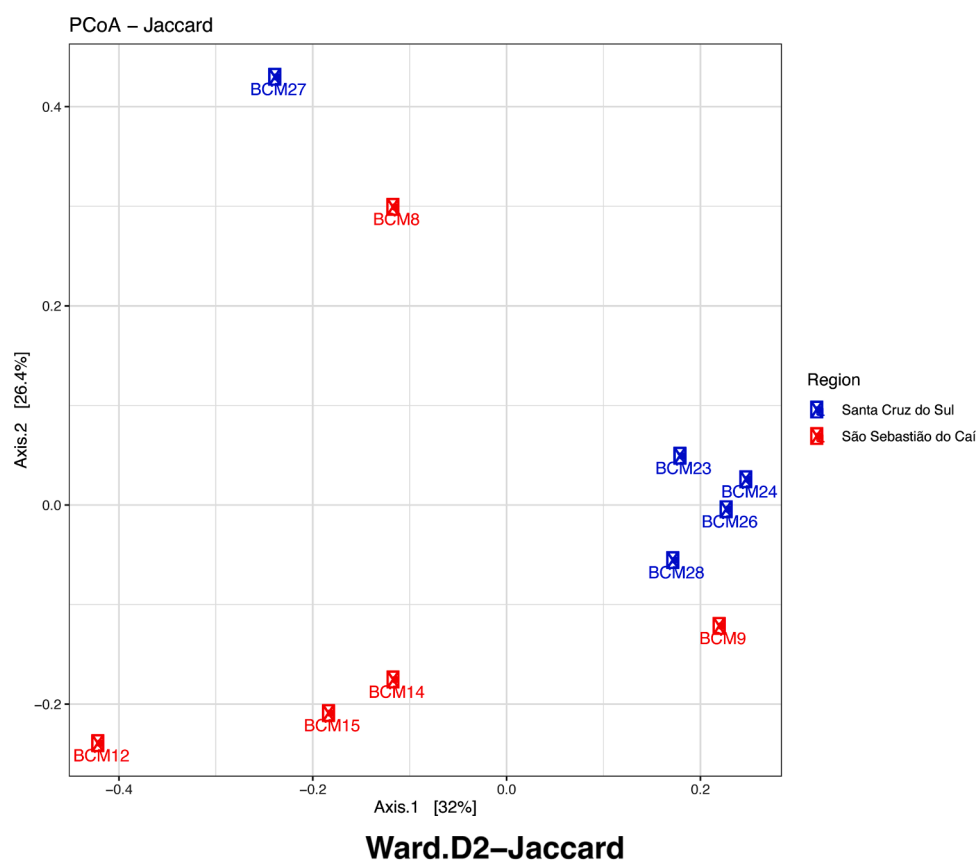


Fig. 3. (continued).

KEGG pathways. We hypothesized that functional genes involved in xenobiotic biodegradation, bacterial infectious diseases, the metabolism of terpenoids and polyketides, and the biosynthesis of secondary metabolites might be associated with anthropogenic habitats, host protection against infection, proximity to humans, and diet (Fig. 4B) (Supplementary Table 2).

Discussion

Microbiomes are multilayered, interconnected networks of microbes and their genes, which interact in time and space to produce a well-functioning host (Björk et al., 2019). The composition and diversity of fecal microbiota are shaped by many factors, including the host's evolutionary history (Youngblut et al., 2019), lifestyle (Zhong et al., 2019), diet (Barone et al., 2018), and social interactions (Dill-McFarland et al., 2019).

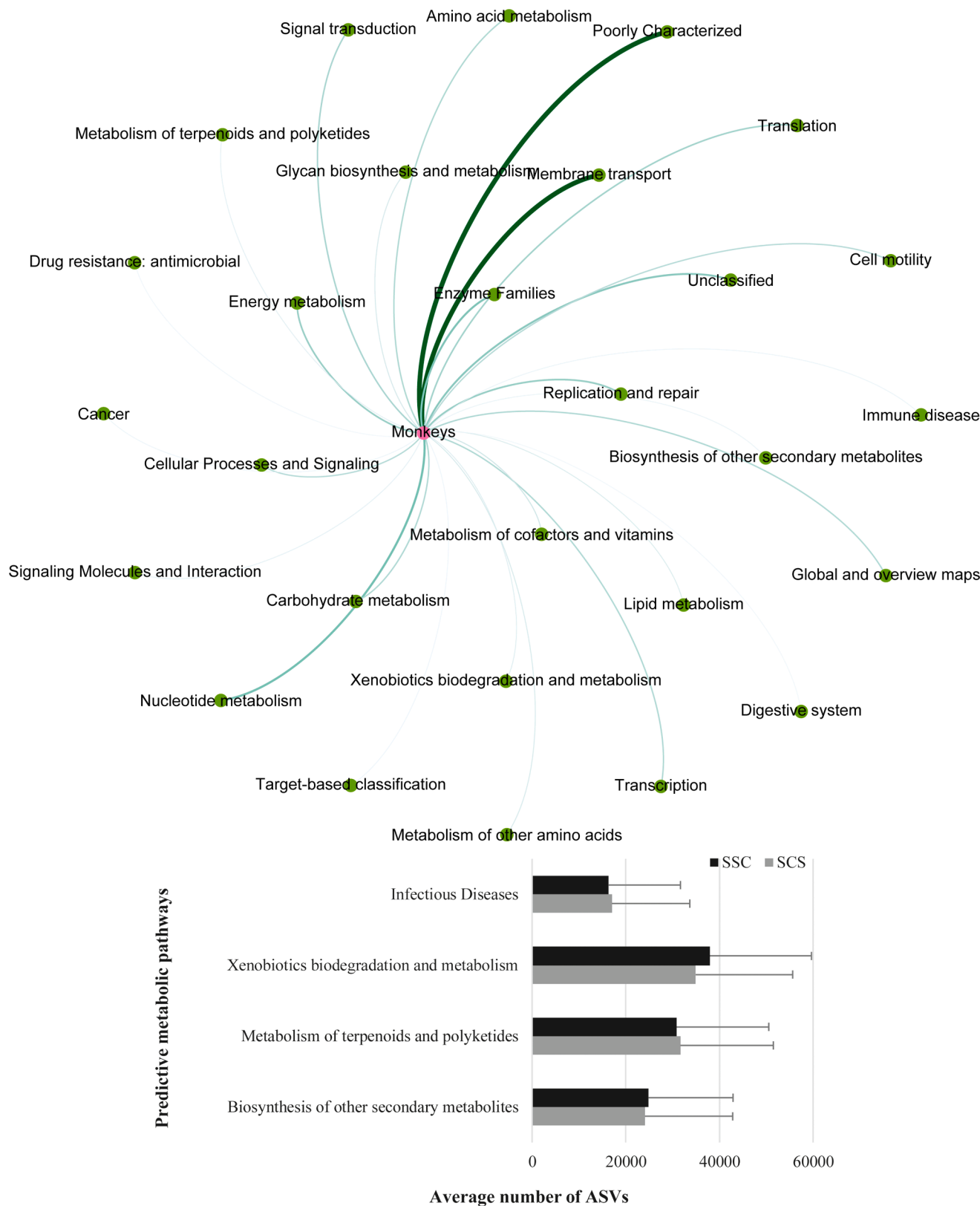


Fig. 4. Predictive metabolic pathways present in the fecal microbiome of wild black capuchin monkeys. Increasing relative abundance of the main predicted metabolic pathways in the fecal microbiota of wild black capuchin monkeys based on the KEGG. Functional predictions were conducted using PICRUST2. (A) Network-wide analysis of co-occurrence dynamics between bacterial taxonomy in fecal microbiota of wild black capuchin monkeys and main predicted metabolic pathways. The thickness and edges colors are concordant with phyla relative abundance in fecal microbiota (dark green are the higher values; pink nodes are the monkeys; green nodes the phyla and metabolisms). (B) KEGG pathways can be seen in the bar plot. Y: Predictive metabolic pathways; X: Average number of Amplicon Sequence Variants – ASVs.

In the present work, no significant differences in the alpha-, beta-, and functional diversity of microbial communities were showed between two distinct populations of wild black capuchin monkeys living in forest fragments of the Atlantic Forest biome. This similar bacterial community composition among individuals from the same groups might be associated with the social behavior of black capuchin monkey populations, such as autocoprophagy and allocoprophagy (Prates and Bica-Marques, 2005). Recently, our group evaluated the genetic similarity of *Enterococcus* species isolated from the fecal and oral samples of wild black capuchin monkeys from SCS using random amplified polymorphic DNA (RAPD) markers. According to the RAPD analysis, the inter- or intra-transmission of enterococci among monkeys was observed, suggesting that autocoprophagy/allocoprophagy and affiliative behaviors might have an important role in shaping and maintaining the enterococci in wild monkeys (Grassotti et al., 2021). Beta diversity also showed no significant difference between the two populations. Although these populations are separated by more than 100 km, their habits are very similar. Notably, a study evaluating the fecal microbiomes of primates from Uganda revealed that microbiomes are strongly associated with host species, and differences in geographic location did not alter this pattern (Mccord et al., 2014).

Proteobacteria was the major phylum in the fecal microbiota of wild black capuchin monkeys. Our results are consistent with those of other studies describing a high abundance of Proteobacteria in the fecal samples of wild and captive marmoset monkeys (*Callithrix* sp.) from

Brazil (Malukiewicz et al., 2019) and wild black-and-white ruffed lemurs (*Varecia variegata*) from southeastern Madagascar (Donohue et al., 2019). However, our results differ from those of other surveys conducted on wild and captive non-human primates (Fig. 5; Supplementary Table 1) that showed a predominance of Firmicutes and/or Bacteroidetes in the gut/fecal microbiota (Artim et al., 2019; Asangba et al., 2019; Bornbusch et al., 2019; Clayton et al., 2019; Dettmer et al., 2019; Duan et al., 2019; Garber et al., 2019; Grant et al., 2019; Grieneisen et al., 2019; Hale et al., 2019; Koo et al., 2019; Lee et al., 2019; Ortiz et al., 2019; Rendina et al., 2019; Adriansjach et al., 2020; Chong et al., 2020; Li et al., 2020; Wei et al., 2020; Wu et al., 2020).

Proteobacteria is the most diverse bacterial phylum and commonly occurs in healthy mammalian gut microbiota (Shin et al., 2015; Moon et al., 2018). The dominance of this phylum is a marker for an unstable microbial community (dysbiosis) in humans and a potential diagnostic criterion for disease (Shin et al., 2015; Rizzatti et al., 2017). The dominance of Proteobacteria in the fecal samples of wild black capuchin monkeys may be associated with anthropogenic activities in the wild habitat of this species, which may affect its diet and/or foraging behavior. Regarding anthropogenic actions, human-monkey interactions have been well documented across several primate species (Back et al., 2019; Morrow et al., 2019). The wild populations of robust capuchin monkeys evaluated in this study live in forest fragments adjacent to urbanized areas and are in close contact with humans, who often feed these animals human foods (e.g., fruit, bread, candy, snacks,

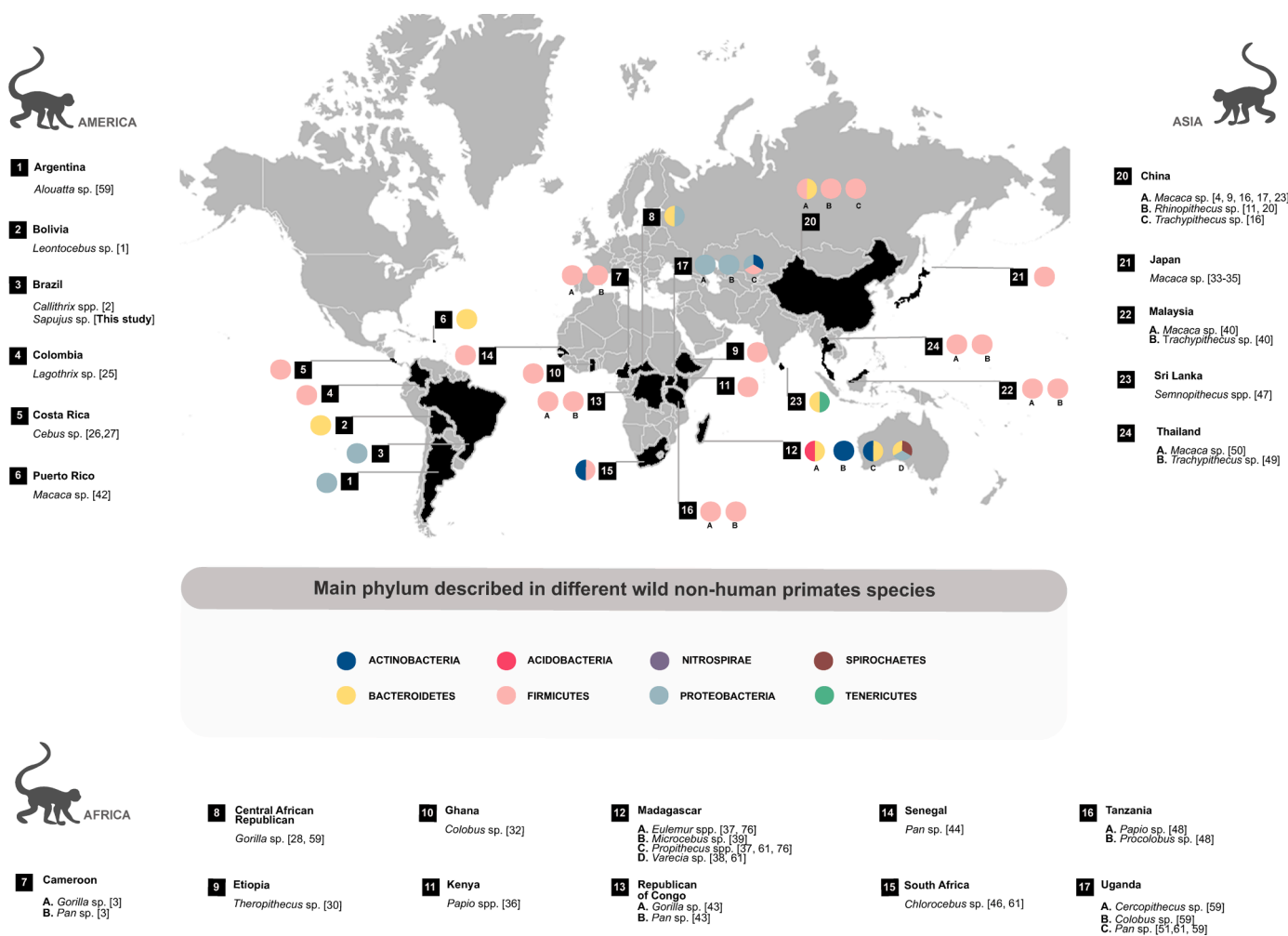


Fig. 5. Map showing the studies were published around the world examining microbial communities of non-human primates from 2019-2021. Papers counted according to the location of the non-human primate. Evaluated only papers with wild animals. Phylum indicators expressed in colors related to qualitative taxonomic diversity. Elaborated by the author.

and meat) (Wenzel and Quadro, 2012). This type of food, which is mainly provided by human visitors, has low nutritional value and high fat content (e.g., bread, candy, and snacks). As shown by Donohue et al. (2019), Proteobacteria was dominant in the fecal microbiota of wild black-and-white ruffed lemurs inhabiting a site accessible to tourists in Ranomafana National Park in Madagascar. The authors suggested that higher visitor presence and potentially increased stress contributed to this group of black-and-white ruffed lemur showing distinct microbial patterning when compared to other groups. Proteobacteria was also the most abundant phylum in marmosets (*Callithrix*) with and without hybrid status (Malukiewicz et al., 2019). The authors reported that marmosets (which have an exudivorous diet) that received food composed of protein, vegetables, fruits, and carbs had 76% more Proteobacteria in their gut microbiota than those without hybrid status. Notably, high fat intake can stimulate proteobacterial inscription in humans (Méndez-Salazar et al., 2018).

Simultaneously, the diet and feeding behavior of the wild black capuchin monkeys in this study may have also contributed to the predominance of Proteobacteria in the fecal samples. In the wild, robust capuchins eat exotic and endemic plants, flowers, and fruits as well as invertebrates and small vertebrates (Ferreira et al., 2002; Ludwig et al., 2005; Rímoli et al., 2008; Izar et al., 2012; Falótico et al., 2018). Lambais et al. (2006) reported that Proteobacteria is predominant in the phyllosphere of endemic tree species of the Brazilian Atlantic Forest, such as the catuaba or catigua (*Trichilia catigua*), red catuaba (*Trichilia clausenii*), and gabioba (*Campomanesia xanthocarpa*). These trees produce edible fruits and provide an important resource for local fauna such as wild black capuchin monkeys (Putzke et al., 2016). Van der Heide et al. (2012) showed that *T. catigua* was recorded in the diets of owl monkeys (*Aotus azarai*) in Chaco, Argentina. The ingestion of insects might also contribute to high levels of Proteobacteria in the fecal samples of wild black capuchin monkeys since this phylum has been reported as dominant in insect gut microbiota (Yun et al., 2014). Thus, the predominance of Proteobacteria may be associated with the plants and invertebrates that they collect and eat. Furthermore, robust capuchin monkeys use stone tools to access a variety of foods, which allows this species to diversify its diet and helps it easily adapt to a diverse range of environments. Furthermore, soil on the stones used by capuchin monkeys might be a source of Proteobacteria for the fecal bacterial community since this phylum is predominant in soils of the Atlantic Forest (Bruce et al., 2010).

The functional profiles of the microbiome data generated in this study were examined using PICRUSt2. Although it has been widely used in works related to the gut microbiomes of non-human primates (Clayton et al., 2019; Duan et al., 2019; Garber et al., 2019; Rendina et al., 2019; Chen et al., 2020a; Chong et al., 2020; Huan et al., 2020), these results are only considered putative and suggestive. The inference of putative microbial functional pathways from fecal microbiomes suggests pathways with positive effects on the health and conservation of monkeys facing anthropogenic environmental degradation, dietary shifts, and infections (e.g., the degradation of xenobiotics (pesticides), biosynthesis of secondary metabolites, and plant metabolism of terpenoids and polyketides). Pathways potentially related to pesticide degradation (e.g., atrazine, chloroalkane, chloroalkene, chlorocyclohexane, naphthalene, ethylbenzene, fluorobenzoate, nitrotoluene, polycyclic aromatic hydrocarbons, toluene, and xylene) were observed in our study (Supplementary Table 2). According to Pignati et al. (2017), Brazil's Rio Grande do Sul state planted 8.5 million hectares and consumed 134 million liters of pesticides. The forest fragments evaluated in our study are adjacent to areas of tobacco, maize, and citrus production. These crops require the application of pesticides, including triazine and organophosphates (Faria et al., 2014; Mendes et al., 2019). The use of pesticides in the agricultural production process has brought a series of disorders and modifications to the environment due to the contamination of human communities and/or its accumulation in biotic segments and abiotic ecosystems. Notably, wildlife can be impacted by

pesticides through their direct or indirect application (Berny, 2007). The ability of the fecal microbiome to degrade xenobiotics such as herbicides (e.g., atrazine) and pesticides (e.g., ethylbenzene) may have a beneficial effect on wild capuchin monkeys' resilience to the human-impacted environment.

Another striking observation in the fecal community was the biosynthesis of several predicted secondary metabolite biosynthetic pathways involved in the production of ansamycins, butirosin, neomycin, novobiocin, streptomycin, tetracycline, and vancomycin (Supplementary Table 2). The occurrence of the Actinomycetaceae, Streptomycetaceae, and Bacillaceae families in fecal microbiota could be related to these results. Secondary metabolism in these communities might be associated with the environmental resistome and thus host infection protection. Ansamycin and tetracycline biosynthesis in the gut communities of wild black capuchin monkeys might have corroborated with the selection of rifampicin- and tetracycline-resistant enterococci strains isolated from the rectal cavities of monkeys from these populations in previous studies by our group (Grassotti et al., 2018). Finally, the plant metabolism of terpenoids and polyketides pathways involved in the degradation of geraniol, limonene, and pinene (Supplementary Table 2) may be associated with the plants, flowers, and fruits eaten by monkeys in the Atlantic Forest. However, little is known about the biosynthesis of gut microorganisms in monkeys, which limits our understanding of the functionality of microbiomes. Therefore, further studies are essential to discover their underlying functions and mechanisms.

Furthermore, the communities of bacteria associated with infectious disease transmission were also observed in KEGG pathways, which suggests a negative effect on monkey health. Senghore et al. (2016) inferred multiple anthropogenic transmissions of *Staphylococcus aureus* from humans to green monkeys (*Chlorocebus sabaeus*) in Africa. Recently, dos Santos et al. (2020) identified human/animal-related viruses such as Anelloviridae, Herpesviridae, Parvoviridae, Papillomaviridae, Iridoviridae, Astroviridae, Poxviridae, Baculoviridae, and Genomoviridae in oral samples from the same individual wild black capuchin monkeys from SCS evaluated in this study. The study also revealed the presence of sequences that share sequence identity with a human-related virus (i.e., Human Gammaherpesvirus 4) and a marmoset-related virus (i.e., *Callitrichine herpesvirus*). Additionally, Campos et al. (2020) also found the presence of rabies virus-neutralizing antibody in one individual of wild black capuchin monkey from SCS. The proximity between humans and wild animals has been associated with zoonotic diseases such as HIV (Peeters et al., 2002; Aghokeng et al., 2010) and the recent SARS-CoV-2 (Andersen et al., 2020). One health approach related to health as an outcome of human-environment systems is known as "health in social-ecological systems" (Zinsstag et al., 2012). Wildlife maintains balance in ecosystems, which is essential to the health of both humans and animals. Due to international concerns, it is essential to maintain microbiological control over wild animals. Therefore, one way to predict and prevent future zoonoses is through the study of the animal microbiome.

One limitation of our study is the low number of animals sampled, which is due to the difficulty of obtaining samples from wildlife. Notably, capturing and handling wild animals requires specialized equipment, the consideration of animal welfare concerns (regardless of the reason for capture), and the efforts of experienced biologists and wildlife technicians to plan and study suitable capture methods. Considering these points, the number of animals captured in the present study equivalent to 10% of the flock. However, because of the small number of subjects, the results should be interpreted with caution.

Conclusion

Wild black capuchin monkeys are endemic to the Atlantic Forest biome in Brazil and the Argentinean provinces of Iguazú and Misiones. They are listed as near threatened mainly due to population reduction

linked to habitat degradation and anthropogenic actions. This is the first study comparing fecal microbiota composition across Brazilian wild black capuchin monkeys living in the Atlantic Forest biome. We detected striking differences in fecal microbiome richness, evenness, and bacterial composition when compared to monkey microbiomes worldwide. Overall, the phylum Proteobacteria and Firmicutes were the most abundant in wild black capuchin monkeys. This similarity being observed in both groups may be due to their feeding strategies, which could be influenced by the Brazilian biome's characteristics and/or human actions in the natural environment. These factors could reduce foraging by this species, which is reflected in their fecal bacterial community.

Interestingly, metabolic prediction revealed pathways in the fecal microbiome that are associated with xenobiotic biodegradation and metabolism, terpenoid and polyketide metabolism, and the biosynthesis of other secondary metabolites. This might suggest positive effects on the health and conservation of monkeys facing anthropogenic disturbances in their environment. Notably, the presence of infectious diseases associated with microorganisms is also an important factor. Therefore, protective measures or training to reduce exposure to personal hazards are important, especially when food is being supplied by people. These results suggest the potential for hygiene measures to mitigate the negative impacts of contact between humans and monkeys to optimize the health of wild black capuchins. Ultimately, this work advances current knowledge of the fecal microbiome of this species while contributing to a better understanding of monkey health and informing conservation measures. However, more research is required to fully understand the fecal microbiome of this species and those of other non-human primate species inhabiting the Atlantic Forest biome, such as *Leontopithecus* spp., *Alouatta* spp., *Callicebus* spp. and other *Sapajus* species. Therefore, the present study provides information about the bacterial population and metabolic functions present in fecal microbiota, which contributes to a better understanding of the ecology and biology of black capuchin monkeys living in forest fragments within the Atlantic Forest biome in Brazil.

Declaration of Competing Interest

None to be declared.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2021.100048](https://doi.org/10.1016/j.crmicr.2021.100048).

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