Antibiotic-producing Micrococcales govern the microbiome that inhabits the fur of two- and three-toed sloths

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Summary

Sloths have a dense coat on which insects, algae, and fungi coexist in a symbiotic relationship. This complex ecosystem requires different levels of control, however, most of these mechanisms remain unknown. We investigated the bacterial communities inhabiting the hair of two- (*Choloepus Hoffmani*) and three-toed (*Bradypus variegatus)* sloths and evaluated their potential for producing antibiotic molecules capable of exerting control over the hair microbiota. The analysis of 16S rRNA amplicon sequence variants (ASVs) revealed that the communities in both host species are dominated by Actinobacteriota and Firmicutes. The most abundant genera were *Brevibacterium, Kocuria/Rothia, Staphylococcus, Rubrobacter, Nesterenkonia, and Janibacter*. In addition, we isolated nine strains of *Brevibacterium* and *Rothia* able to produce substances that inhibited the growth of common mammalian pathogens. The analysis of the biosynthetic gene clusters (BCGs) of these nine isolates suggests that the pathogen-inhibitory activity could be mediated by the presence of siderophores, terpenes, beta-lactones, Type III polyketide synthases (T3PKS), ribosomally synthesized, and post-translationally modified peptides (RiPPs), non-alpha poly-amino acids (NAPAA) like e-Polylysin, ectoine or nonribosomal peptides (NRPs). Our data suggest that Micrococcales inhabiting sloth hair could have a role in controlling microbial populations in that habitat, improving our understanding of this highly complex ecosystem.

Introduction

Almost all mammals have fur or other hair-related structures that help them adapt to their habitats. Hair usually provides thermal regulation and camouflage (Dawson et al., 2014) also it has been

related to some communication and mating processes (Buffoli et al., 2014). Beyond their functional role for animals, hair constitutes a habitat for multiple microorganisms on which they coexist and associate in complex ways with their host (Chen et al., 2018). In mammals, due to their anatomical proximity, it is difficult to separate the microbiome associated with skin and fur, and the vast majority of studies refer to the skin microbiome (Belheouane et al., 2020; Chen et al., 2018; Council et al., 2016; Ross et al., 2019). For example, a high microbial diversity has been reported in human skin (Byrd et al., 2018), and it has been shown that it presents a high proportion of symbionts that control the population of pathogenic bacteria through the secretion of multiple compounds such as bacteriocins or fatty acids (Byrd et al., 2018; O'Sullivan et al., 2019). For its part, the hair microbiota in humans has been shown to have a similar composition to skin samples (Polak-Witka et al., 2020; Saxena et al., 2021). The most abundant genera found in hair follicles correspond to *Cutibacterium acnes* (previously known as *Propionibacterium acnes*), *Staphylococcus* sp., *Corynebacterium*, *Streptococcus*, *Acinetobacter*, *Prevotella* and *Malassezia* (Lousada et al., 2021; Polak-Witka et al., 2020; Watanabe et al., 2021), however, it is important to mention that this microbiota is influenced by multiple factors including demography, use of cosmetics products or oils and genetics (Brinkac et al., 2018).

The human skin (and fur) microbiome is the most studied; the microbiome present in the skin and hair of other mammals and their possible functions are poorly understood or remain completely unknown. Understanding these microbe-mammalian hair relationships is important not only from an ecological perspective but also because we could reveal microorganisms, enzymes or small molecules of biotechnological interest. A global analysis of multiple animals' skin microbiome, based on 16S rRNA amplicon sequencing revealed that the skin of most mammals differs significantly from the human skin microbiome and resembles soil microbiome in most cases, which is probably a

consequence of the constant environmental exposure of their fur. Also, a phylosymbiosis of the microbiota was found among the different mammals' clades. Bacteria belonging to *Staphylococcus*, *Corynebacterium,* and *Micrococcus* genera are reported as recurrent members on the skin and fur of mammals (Kloss et al., 1976; Ross et al., 2019). In non-human primates, it was revealed that even though members of the *Staphylococcus* and *Corynebacterium* genera were present, other phylotypes such as *Anaerococcus* and *Prevotella* are an abundant part of the microbial community (Council et al., 2016). Furthermore, on livestock animals, it has been shown that the skin microbiome has an important prevalence of Bacteoidetes, Chloroflexi, and Proteobacteria (Ross et al., 2018; Ross et al., 2019). The role of these phylotypes in the animal's physiology is unclear. Nevertheless, it has been postulated they can be involved in protection from pathogenic bacteria via competition or maturation of the immune system (Bradley et al., 2016; Rodrigues Hoffman, 2017).

Sloths are mammals whose skin and hair microbiota is partially known (Higginbotham et al., 2014; Pauli et al., 2014). Sloths are mammals adapted to live in the canopy layer of the rainforest. These animals are characterized as being solitary with parsimonious movements and a relatively low metabolic rate compared to other mammals (Falconi et al., 2015; Taube et al., 2008). They have a low body temperature, making them intolerant to sudden variations in temperature; for this reason, they spend most of their time sunbathing and feeding (De Oliveira et al., 2014). There are a total of six different sloth species divided into two genera: *Bradypus* (two-toed sloths), with four species, and *Choloepus* (three-toed sloths), with two species (Dill-McFarland et al., 2016; Mendoza et al., 2015). In Costa Rica, only two species are present *B. variegatus* and *C. hoffmanni* (Vaughan et al., 2007). These two species were declared in August 2021 as a national symbol in the country due to their importance in touristic activities and their ecological roles (see Figure 1).

Sloth fur is a dense coat on which macro- and microorganisms coexist (see Figure 1). It is usually covered with green algae from the genus *Trichophilus,* which helps sloths camouflage from predators (Suutari et al., 2010). Also, a moth classified as *Cryptoses* lives on sloths' hair and is in charge of the nitrification process that supports the growth of *Trichophilus* (Waager and Montgomery, 1976). Previously, a study that focused on culturable fungi that inhabit two-toed sloth's hair revealed that these fungi secrete multiple compounds that inhibit the growth of numerous bacteria and parasites such as *T. cruzi* (Higginbotham et al., 2014). Even though sloths fur has been proved to be a complex habitat with various organisms occupying it, little information exists about its bacterial community, functions, and possible roles as symbionts of these mammals. In this work, we characterize the bacterial community inhabiting the hair of both sloth species from Costa Rica (*Choloepus hoffmani* and *Bradypus variegatus*) (see Figure 1). Additionally, we intend to explore the possible roles of these bacteria in the pathogen control of the microbial community on sloth hair.

Experimental Procedures

Sampling and permits

All samples of sloth hair were obtained from the Sloth Sanctuary (http://www.slothsanctuary.com) in Cahuita, Limon (9°47'58.5"N 82°54'53.1"W) in February 2020. The sanctuary shelters around 250 individuals of *Bradypus variegatus* and *Choloepus hoffmani* (see Figure 1). The Sloth Sanctuary has been operating since 1992 and is dedicated to the rescue, rehabilitation, and research of sloths and conserving their rainforest habitat. All the sloths have come to the sanctuary because they were abandoned by their mothers, or have suffered some kind of accident. In cases where it is possible, the animals are reinserted into their natural habitats, however, many do not have that capacity and

are therefore kept in the refuge. All sloths are kept in cages which can be either alone or with partners of the same species. In the case of shared cages, only one sloth per cage was sampled. Hair samples were taken from the back of the sloth usinors and then stored in sterile 50 mL conical tubes. We obtained two subsamples from 13 individuals of *Bradypus* and 15 individuals of *Choloepus*. The names, code, years in the refuge, and other related characteristics of each sampled animal are shown in Table S1. Samples for total DNA extraction were stored at -20 °C until processed. The samples for bacterial isolation were immediately transported to the laboratory and processed as described below. Permits to sample were obtained from the Institutional Commission of Biodiversity of the University of Costa Rica (resolution Nº 253) and Judy Avey-Arroyo, the property owner.

Scanning electron microscopy

Hairs of *B. variegatus* and *C. hoffmani* ~5 mm long were fixed with osmium tetroxide vapor (4%, in 0.2 M sodium phosphate buffer, pH 7.2). The hair fragments were placed in 2-cm-diameter plastic lids, which were kept inside a 15-cm-diameter glass Petri dish containing filter paper moistened with the osmium solution for 48 hours in a hood of extraction. After this time, the material was removed from the Petri dish and left to dry in the fume hood for 12 hours. The hairs were placed on an aluminum base containing double-sided carbon conductive tape and plated with gold in a Denton Vaccum Desk V ion spreader (20mA, 3 minutes). Subsequently, the hairs were observed in scanning electron microscopy (JEOL JSM 6390LV) at 20 KV.

Total DNA extraction and sequencing

For total DNA extraction of sloth hair, the samples were proceeded as described by Rojas-Gätjens (2021) with some modifications. Briefly, hair samples were cut with sterile scissors into smaller pieces. A total of 300-500 mg of hair per sample was extracted with a DNA isolation kit (PowerSoil®, MoBio, Carlsbad, CA, USA) as described by the manufacturer. Cell lysis was accomplished by two steps of bead beating (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) for 30 s at 6.0 m s^{-1}. For the construction of the microbial amplicon library (16S rRNA), the V4 hypervariable region was PCR-amplified with universal primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Carporaso et al., 2010). The PCR-generated products were subjected to a 250 nt paired-end sequencing (Novoseq, Novogene).

Bioinformatic and statistical analysis

Amplicon sequencing data was analyzed using the DADA2 pipeline (version 1.12), as described by Rojas-Gätjens et al. (2021). Briefly, reads were quality-filtered, deduplicated, and amplicon sequence variant (ASV), a higher-resolution analog of the traditional operational taxonomic unit (OTU), was inferred. The chimeric sequences were removed. This process resulted in an ASV table, which records the number of times each exact amplicon sequence variant was observed in a sample. ASVs were taxonomically assigned by comparing sequences against the SILVA reference database v138 (Quast et al., 2013) and then curated by comparing sequences against the GenBank database of NCBI. Global singletons, doubletons, and sequences classified as Eukarya, Chloroplast, and Mithocondria were removed. The average number of sequences per sample obtained was 132265, ranging from 91415 to 170677. For estimation of the core microbiome of both sloths, we use the package R package microbiome (Lahti and Shetty, 2018). The core microbiome was defined with a prevalence of 50% and abundance of 1% according to Lahti and Shetty (2018). The raw sequencing

168 data were deposited in the sequence-read archive (SRA) of Genbank under the Bioproject ID
169 PRJNA804868
170
171 The statistical analysis and their visualization were performed with R statistical program (R-core-
172 Team 2019) and the Project Jupyter notebook interface. The package Vegan v2.5-6 (Oksanen et al.
173 2019) was used to calculate alpha diversity estimators (Observed abundance, Shannon index, and
174 Simpson index) and Non-metric multidimensional scaling analysis (NMDS). Wilcoxon test was
175 performed for comparison of the alpha diversity estimators between hosts (*Choloepus hoffmani* and
176 *Bradypus variegatus*). Data tables with the ASV abundances were normalized into relative
177 abundances and then converted into a Bray–Curtis similarity matrix. The effects of the hosts and sex
178 on the bacterial community composition were evaluated with non-parametric multivariate analysis of
179 variance (PERMANOVA) and pairwise PERMANOVA (adonis2 function with 999 permutations). The
180 effect of the time they present in captivity was evaluated using the envfit function developed on the
181 package Vegan v2.5-6 (Oksanen et al. 2019).
182
183 *Isolation of antibiotic-producing bacteria*
184
185 Samples were processed as described by Cambronero-Heinrichs (2019). Briefly, samples were
186 diluted in PBS 1X (Na_2HPO_4 0.01 mol L^{-1}, KH_2PO_4 0.0018 mol L^{-1}, NaCl 0.137 mol L^{-1}, KCl 0.0027
187 mol L^{-1}) employing a 1:1 sample weight/volume ratio and then serially diluted until 10^{-3}. Dilutions were
188 pre-incubated (60°C for 10 min) for avoiding the growth of Enterobacteria. Dilutions were plated on
189 oatmeal agar (Jensen et al., 2005) supplemented with nalidixic acid (25 mg ml^{-1}) and
190 cycloheximide/clotrimazole (50 mg ml^{-1}). Plates were incubated for three months (30°C) and

examined every week. All bacteria were transferred to ISP2 medium (Pridham et al., 1957). All isolates were preserved (−80°C, 15 % glycerol).

Screening of antimicrobial activity

The ability of isolates to inhibit bacterial growth was evaluated by the agar disc diffusion method described by Cambronero-Heinrichs (2019). Since there are no known possible pathogens on sloth skin, we decided to perform antibiotic activity tests against ATCC model microorganisms such: *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ATCC25923, *Candida albicans* ATCC10231, and *Bacillus subtilis subsp. spizizenii* ATCC6336. In addition, we included in the test set other available microorganisms known to be pathogenic in mammals (i.e. *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Kleibsella pneumonie*, *Serratia marcescens),* as well as some genera of fungi that were isolated from the hair of sloths previously (i.e. *Cytospora sp., Fusarium sp., Colletrichum sp., Hypocrea sp., Trichoderma sp*) (Higginbotham et al., 2014).

Bacteria and yeast pathogens strains were individually inoculated in either Luria–Bertani agar or potato dextrose agar (OD = 0.1, from overnight cultures). A piece of agar (diameter 6mm) with confluent growth of each actinobacterial isolate (2-week-old cultures in ISP2) was cut and placed on the surface of the medium, with bacterial growth uppermost. A Whatman® cellulose filter paper inoculated with kanamycin (5 uL, 5 mg/mL; 50 mg/mL for *P. aeruginosa*) or clotrimazole (5 mg/mL) was used as a positive control. A filter without any substance was used as the negative control. The plates were incubated for 24h at 37 °C for *E. coli*, *P. aeruginosa*, *S. aureus*, *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Kleibsella pneumonie*,

215 *Serratia marcescens,* and *C. albicans*, and 30° C for all others. Every experiment was performed in
216 duplicate. Inhibition halos were measured in centimeters, and the results were ranked by size (see
217 Table 1).
218
219 *Identification of bacterial isolates*
220
221 For identification of the antibiotic-producing isolates, each one was grown individually (ISP2 agar, 1-4
222 weeks). Then, DNA was extracted according to the protocol described by Chun and Goodfellow
223 (1995). DNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher,
224 USA). Polymerase chain reaction (PCR) protocols described by Cambronero-Heinrichs et al. (2019)
225 to amplify 16S rRNA with primers 27F and 1492R were used. PCR products were visualized on
226 agarose gels (1%) stained with Gel Red (Biotium Inc., USA). The PCR products were sequenced
227 using BigDye Terminator v3.1 cycle sequencing kit and cleaned with BigDye XTerminator purification
228 kit (Applied Biosystems, USA). Products were analyzed on a 3130xl Genetic Analyzer (Applied
229 Biosystems, USA). Forward and reverse sequences were assembled using Bioedit. The obtained
230 consensus sequences were compared against 16S rRNA bacterial/archaeal database of the NCBI
231 using BlastN. All sequences were deposited on the Genbank under accessions OM638436-
232 OM638444.
233
234 *Biosynthetic gene clusters (BGCs) analyses of antibiotic-producing isolates*
235
236 For biosynthetic gene clusters (BCGs) analyses, the nine bioactive isolates were grown on ISP2
237 media (5 days at 30 ºC). Specimens classified within the same genera were combined in sterile
238 microcentrifuge tubes with a difference in OD600 of 1:2 to achieve a good binning of the genomes

239 according to their abundance. DNA was extracted using a DNA isolation kit (PowerSoil®, MoBio,
240 Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quality checked and
241 subjected to a 250 nt paired-end sequencing (Novoseq, Novogene). The raw sequences from this
242 study were deposited in the GenBank database BioProject ID PRJNA804868
243
244 For the construction of the Metagenome Assembled Genomes (MAGs) sequences were quality
245 filtered using bbmaptools (function bbduck) and then quality checked with FastQC. Sequences were
246 assembled with MEGAHIT (Li et al., 2016). Only contigs larger than 1500 bp were further analyzed.
247 The open reading frames were mapped using Prodigal v2.6.3 (Hyatt et al., 2010) and the single-copy
248 core genes were identified with HMMER v3.3 (Johnson et al., 2010). The taxonomy of the SCG
249 genes was estimated using DIAMOND (Buchfink et al., 2015). Manual binning was performed using
250 Anvio v6.2. A bin was considered a MAG if it presented 100% completeness and >5% redundancy.
251 Four bins were obtained from which two were classified as MAGs. Phylogenetic analysis of the four
252 bins with closely related genomes (see table 2 for Genbank accessions) was performed by using 71
253 single-copy core genes (a curated list of genes available in Anvi'o phylogenomic workflow
254 (https://merenlab.org/tutorials/infant-gut/#chapter-iii-phylogenomics) shared among the different
255 genomes. Sequences were concatenated and further aligned using MUSCLE v3.8.1551 (Edgar,
256 2004). Phylogenetic relationship was estimated using FasTree v2.1 (Price et al., 2009).
257
258 BGC analyses were conducted employing antiSMASH 5.0 (Blin et al., 2019) with default relaxed
259 detection parameters that identify well-defined complete clusters and partial clusters missing one or
260 more functional parts. We applied the following criteria for determining a known BGC: 100% of its
261 genes have a significant BLAST hit to the corresponding genes of a known compound in the MIBiG
262 database.

Results and Discussion

SEM reveals a high density of cocci-and bacilli-shaped microorganisms in sloths hair.

Similar to the results obtained by Wujek and Cocuzza (1986), we observed through a scanning electron microscope (SEM) that the hair of sloths of the genus *Choloepus* has a series of longitudinal furrows and an average width of 138 ± 13 μm. On the other hand, the hair of sloths of the genus *Bradypus* is smoother, with irregular fissures throughout the entire hair and an average width of 261 ± 36 μm. Hair samples from both *Choloepus* and *Bradypus* (Fig. 2), showed a high density of cocci- and bacilli-shaped microorganisms. This high density of microorganisms was more noticeable in the hair of the sloths of the *Choloepus* species, which could be explained by the morphology. As observed in the SEM micrographs, the microbial communities are preferentially established within the furrows of the hair of the genus *Choloepus*. Presumably, this location can be favored by a lower exposure to mechanical stress, and greater availability of water and nutrients with respect to the ridges of the hair. Most of the microorganisms observed in those habitats correspond to coccoid bacteria, nevertheless, some hyphae-like structures and rod-shaped bacteria were present in less abundance. In *Bradypus* hair, a lower microbial density was observed and its location was not necessarily associated with the fissures throughout the entire hair. In *Bradypus,* it is also possible to observe coccoid bacteria, as on the hair of *Choloepus*.

Since the sloths live in the refuge, the development of the characteristic greenish color in their fur was not observed, typically associated with the presence of the green algae *Trichophilus*. In the SEM

286 photos, we were not able to determine the presence of cells of this algae either. Its absence is not
287 rare, since the appearance of this algae occurs in wild sloths as a camouflage mechanism, which is
288 not required in individuals living in captivity. Despite the absence of green algae *Trichophilus*, these
289 first observations show that sloth hair is an environment rich in microorganisms. To reveal the identity
290 of these microbial communities we decided to perform an analysis of 16S rRNA gene amplicons.
291
292 *Analysis of 16S rRNA gene amplicons indicates that sloth hair is governed by members of the*
293 *Micrococcales order and shaped by the environment*
294
295 The bacterial community inhabiting the hair of both sloth genera showed high diversity values. The
296 average estimations of the Shannon-index were 3.06 and 2.72 for *C. hoffmani* and *B. variegatus*,
297 respectively (see Figure S1). Wilcoxon test revealed a statistically significant difference (Shannon-
298 index, $p = 0.045$) in the diversity of the microbial communities between the two sloths, in which
299 *Choloepus* showed higher diversity than *Bradypus*; although multiple phylotypes were identified in all
300 samples. In total, 4892 amplicon sequence variants (ASV) were identified in the 28 samples. The
301 most abundant bacterial phyla were Actinobacteriota (10.82-96.44%), Firmicutes (1.60-65.56%),
302 Proteobacteria (0.53-36.75%), and Bacteroidota (0.53-32.81%). Other phyla were detected in lower
303 proportions, such as Acidobacteriota (0-1.91%), Chloroflexi (0.59-2.87%), Cyanobacteria (0-0.59%),
304 Desulfobacteria (0-0.62%), Gemmatimonadota (0.84-3.46%) (see Figure 3A and Table S2).
305
306 At the order level, we observed that the bacterial community inhabiting the hair of both species of
307 sloths was mainly dominated by members of the Micrococcales (see Figure 3B). Within this order, the
308 families Brevibacteriaceae (0.70-75.30%) and Micrococcaceae (2.61-71.23%) were the most
309 abundant (see Figure S2). Members of the Brevibacteriaceae family have been previously isolated

310 from a variety of environments including dairy products (Forquin et al., 2009), poultry skin (Forquin-
311 Gomez et al., 2014), insects (Katı et al., 2010), mural paintings (Heyrman et al., 2004), clinical
312 samples (Roux and Raoult, 2009; Talento et al., 2009), saline sediments (Chen et al., 2016),
313 mammal skin (Collins et al., 1983) and human microbiota (Forquin-Gomez et al., 2014). Most of the
314 organisms found in this family are characterized as being halotolerant with the ability to produce
315 sulfur volatile compounds (Arfi et al., 2003). Members of the Micrococcaceae family are usually
316 associated with the skin microbiota of mammals (Collins et al., 2000), blood cultures (Hansen, 1985),
317 and in various soil samples including marine sediments (Kim et al., 2004). These microorganisms
318 have demonstrated a vigorous metabolism and an exceptional ability to adapt to harsh conditions
319 (Dastager et al., 2014). Additionally, even though some members of both families ie.
320 Brevibacteriaceae and Micrococcaceae have been associated with bacteremia and other infections
321 (Asai et al., 2019; Bal et al., 2015; Kandi et al., 2016; Ramanan et al., 2014), most of the
322 microorganisms belonging to this group are commensals frequently found on the skin and oral
323 cavities of mammals (Collins et al., 1983; Dastager et al., 2014).
324
325 Furthermore, a significant proportion of members of the Staphylococcales (1.08-45.39%) and
326 Rubrobacterales (0.17-44.57%) were found in the hair samples of *Bradypus* and *Choloepus*
327 specimens (Figure 4B). Members of the family Staphylococcaceae are commonly found in the skin of
328 multiple mammals (Lory et al., 2014). Particularly *Staphylococcus aureus* is a widely-known pathogen
329 associated with multiple infections and has become more deadly due to the increase of antibiotic
330 resistance (Hassoun et al., 2017). The prevalence of members of the Staphylococcaceae family in
331 skin samples has been previously reported in multiple mammals, particularly in those mammals that
332 have a strong anthropogenic influence (Ross et al., 2018) which is the case of the sloths sampled,
333 that dwell in a human-controlled refugee. Contrarily to Staphylococcaceae, Rubrobacteriaceae

334 members are characterized for being extremophilic bacteria, which have shown to survive extreme
335 radiation, including gamma radiation (Ferreira et al., 1999). Members of this family are pink-
336 pigmented bacteria commonly isolated from hot springs (Carreto et al., 1996), biodeteriorated
337 monuments (Jurado et al., 2012), marine sponges (Kämpfer et al., 2014) and deep-sea sediments
338 (Albuquerque et al., 2014). The presence of this taxon in sloth hair is probably associated with the
339 high temperatures (the Sloth Sanctuary is located in Cahuita, with an annual temperature range of 21
340 to 31 ºC) and the constant exposure to UV radiation that the sloths endure in their niche (Duan et al.,
341 2017; Gohli et al., 2019; Sun et al., 2018). Nevertheless, further study is required to comprehend the
342 association between these extremophilic microorganisms and their hosts.
343
344 As seen in Figure 3C, abundant ASVs detected in sloth's hair encompass members of the genera
345 *Brevibacterium* (0.70-75.30%), *Kocuria* (0.94-47.17%), *Staphylococcus* (1.01-45.09%),
346 *Brachybacterium* (0.60-31.07%), *Nesterenkonia* (0.04-69.61%) and *Rubrobacter* (0.17-44.57%).
347 These genera are characterized for inhabiting sediments usually with a high salt concentration
348 (Chander et al., 2017; Dastager et al., 2014; Duan et al., 2017; Forquin-Gomez et al., 2014;
349 Stackebrandt, 2014). The Sloth Sanctuary in Cahuita is located just 1.7 km from the Atlantic coast of
350 Costa Rica, where high concentrations of salt in the environment are expected to occur. Therefore, it
351 is not surprising that the microorganisms that inhabit the hair of the sloths in this refuge have a high
352 content of bacteria associated with habitats with high concentrations of salt, thus being a factor that
353 shapes the microbial community. It has also been shown that compounds secreted by the glands of
354 the host can shape the microbial community through the variation of pH, salinity, and osmotic
355 pressure (Byrd et al., 2018; Grice and Segre, 2011; Sanford and Gallo, 2013). The presence of
356 microorganisms associated with saline sediments on our hair samples agrees with previous studies

that showed that mammals' skin microbiome resembles the community from the environment, including soils and sediments (Ross et al., 2018).

Specifically, sequences classified as *Brevibacterium* ASV1 and ASV6 were abundant ASVs particularly in *Bradypus* samples (see Figure 3C). *Brevibacterium* is a highly heterogeneous genus with multiple metabolic capabilities (Forquin-Gomez et al., 2014). It has been shown that this genus usually prefers to metabolize lactate as a source of carbon; lactate has been reported as a secreted compound by sebaceous mammals' glands (Downey and Kealey, 2004). Additionally, this genus can also metabolize amino acids as carbon and nitrogen sources, probably allowing them to adapt well to the highly competitive hair niche on which algae, insects, protozoa, and other bacteria compete for resources (Mounier et al., 2007). Similarly, multiple sequences identified as *Kocuria* were found in samples belonging to both genera (*Choloepus* and *Bradypus*) (ASV3, ASV12, ASV16, and ASV17). *Kocuria* is characterized for mainly being aerobic chemoheterotroph microorganisms (Dastager et al., 2014). It usually expresses polymer degrading enzymes such as esterases (Kaur et al., 2011), and lipases (Reddy et al., 2003), suggesting that they might have the ability to degrade biopolymers found in the skin. A keratinase has also been found in *Kocuria* specimens (Bernald et al., 2006), which probably allow them to degrade the hair in which they inhabit to obtain energy.

Additionally, various abundant ASVs were classified as *Rubrobacter*. As mentioned above, the genus *Rubrobacter* is widely known for its ability to survive radiation and desiccation (Chen et al., 2004). Its resistance to these conditions probably allows members of this taxon to live on the sloth hair which is constantly exposed to UV radiation and desiccation due to the high sodium chloride content. Additionally, ASV classified as *Staphylococcus* were highly abundant, similar to what has been reported in human skin, where *Staphylococcus* and *Propionibacterium* are the main members

of the community (Byrd et al., 2018; Cogen et al., 2008). It has been previously shown that *Staphyloccocus* is part of the normal hair microbiota in humans (Lousada et al., 2021). Even though some species such as *S. aureus* are important pathogens, most of the genotypes belonging to this group are commensal microorganisms that accomplish important processes for maturation of the immune system or secreted bacteriocins-like proteins that inhibit the proliferation of pathogenic bacteria (Nguyen et al., 2017; Newstead et al., 2020; Carson et al., 2017).

Finally, other sequence variants were assigned to the Actinobacteriota genera *Brachybacterium* (ASV5, ASV10, ASV13) and *Nesterenkonia* (ASV4, ASV9, ASV32, ASV29, ASV15). Both of these genera are poorly studied bacteria which, similar to *Brevibacterium* and *Kocuria*, show a great diversity in their niches, which range from plant growth promoters to human opportunistic pathogens (Edouard et al., 2014; Tak et al., 2018; Singh et al., 2016).

The sampled two- and three-toed sloths share almost 50% of the core microbiome in their hair

Further Non-Metric Multidimensional and PERMANOVA analysis revealed that even though similar bacterial phyla were found in *Choloepus* and *Bradypus* hair, a significant difference between the bacterial communities was found (PERMANOVA, $p = 0.001$) (see Figure 4A). No significant difference was found between the sex ($p = 0.18$) or time in captivity ($p = 0.143$) for either species (data not shown). Previous analyses of the skin microbiome of multiple mammals found a specialization along mammal orders (Ross et al., 2018). It is reasonable to think that this specialization may also occur between sloth species. The difference in the microbial community observed could be associated with the physiology of each sloth as the compounds secreted by multiple glands in the skin would shape the microbial community.

Even though there is a significant difference in the microbial community between sloth species (see Figure 6A), our data revealed that the core microbiome of both sloths is similar (see Figure 4B). The core microbiome (according to the definition of Lahti and Shati (2017)) of both sloths consists mainly of members of the Actinobacteriota phylum and bacteria from the *Staphylococcus* genus, particularly of ASVs classified as *Brevibacterium*, *Staphylococcus*, *Kocuria*, *Nesterenkonia*, *Brachybacterium*, *Rubrobacter*, *Janibacter*, *Dietzia,* and *Saccharopolyspora*. The core microbiome of both sloth species in the Sanctuary shares almost 50% of the ASVs (see Figure 6B) which could suggest that these microorganisms exert a functional role in this habitat. Although the sampled sloths are in separate cages, not mixed between species, and the Sanctuary employees avoid handling the animals, the presence of a core microbiome that is shared between the two species must be validated with samples from other regions and with free-living animals. Despite this, our work provides a base and gives the first light of the microbiota that establishes itself in the fur of the sloths.

In an attempt to elucidate the role of the members of the microbiome in both sloth species and considering that many of these microorganisms belong to the phylum Actinobacteriota, we decided to evaluate the presence of bacteria that produce antibiotic molecules. Members of the phylum Actinobacteriota are known for their ability to produce antibiotic molecules, and many of them are found in association with higher organisms establishing symbiotic relationships (Matarrita-Carranza et al., 2021).

Several members of the core microbiome are antibiotic-producing bacteria

To evaluate the capacity of the bacteria inhabiting sloth's hair to produce antibiotic compounds, we isolated Actinobacteriota and evaluated their anti-microbial activity against multiple pathogenic microorganisms (see Experimental procedures). We were able to isolate 54 bacteria of which nine were antibiotic-producing. From these, five were isolated from *Choloepus* hair and four from *Bradypus* hair.

The antibiotic-producing bacteria were classified as either *Brevibacterium sediminis*, *Rothia koreensis,* or *Rothia kristinae*. Interestingly these genera were classified as part of the core microbiome in the microbial community analysis. *Rothia kristinae* and *Rothia koreensis* were only recently classified within the *Rothia* genus through phylogenomic analysis (Nouioui et al., 2018), before 2018 these bacteria were classified within the *Kocuria* genus according to its 16S rRNA sequence (Stackebrandt et al., 1995) suggesting that the absence of *Rothia* genus is a consequence of the close phylogenic relationship between *Rothia* and *Kocuria* that do not allow a clear separation through methodologies. Blastn of abundant ASV classified as Kocuria showed a 100% identity with *Rothia kristinae* or *Rothia koreensis*.

Biological activity was observed against *S. aureus*, *E. coli*, *P. aeruginosa*, *B. Subtilis*. *S. agalatiae* and *Cytospora* sp., however, the antimicrobial activity presented a wide variation even among strains of the same genus (see Table 1). Strains that showed activity against *S. aureus* (*R. koreensis* 10C1, *R. kristanae* 13C8, 9C1, 11C2, 3B2, and 3B6) showed no activity against *S. epidermidis* which is known to be a common symbiont in mammal's skin (Liu et al., 2020). The ability of these isolates to inhibit well-known pathogens growing but not affect closely related symbionts may indicate that these Micrococcales may act as a protective mechanism principally against pathogens on sloth skin. Nevertheless, no inhibition was observed against other well-known pathogens such as *K. pneumonie*,

452 *S. agalactiae,* and *S. saprohyticus*. Notably, *S. agalactiae* presented growth inhibition with isolate
453 3B2 an inhibition on its capacity to perform hemolysis when exposed to 9C1, 11C2, 3B2, and 13C9
454 (data not shown). Additionally, multiple strains presented activity against *Bacillus subtillis* which have
455 been associated with food poisoning (13C8, 9C1, 11C2, 3B2, and 3B6).
456
457 Since many fungi have demonstrated the ability to infect the skin, we attempt to evaluate the
458 antifungal activity of our isolates against fungi closely related to the ones previously found in
459 *Bradypus* fur (Higginbotham et al., 2014) (see Experimental procedures). Three isolates
460 (11C2,13C9, and 13C8) showed antibiosis against *Cytospora* sp. Despite *Cytospora* sp. is not a
461 recognized mammal pathogen, its presence in sloth hair may indicate that these Microccocales have
462 evolved to compete with other microorganisms that occupy a similar niche.
463
464 Furthermore, multiple isolates showed the ability to inhibit other Gram-negative bacteria such as
465 *Pseudomonas aeruginosa* and *Escherichia coli* (see Table 1). *P. aeruginosa* is an opportunistic
466 pathogen that has shown an intrinsic resistance to a variety of antibiotics (Gellatly et al., 2013; Qiu et
467 al., 2009; Thi et al., 2020). Nowadays, *P. aeruginosa* strains resistant to almost all antibiotics (Molina-
468 Mora et al., 2020) have been isolated and represent a significant health problem; therefore, isolates
469 that show the ability to inhibit its growth are interesting for future research to identify the compounds
470 responsible for this activity.
471
472 *BGCs found in antibiotic-producing bacteria are poorly studied but well conserved among strains of*
473 *Micrococcales.*
474

475 When analyzing the Biosynthetic Gene Clusters (BGCs) in the assembled genomes, we were able to
476 manually bin the sequences into four bins (see Table 2). Bins were classified as *Rothia koreensis*
477 (Bin 3), *Rothia kristinae* (Bin 1 and Bin 2), and *Brevibacterium* (Bin 4). Furthermore, a comparison
478 with closely related genomes obtained from the Genbank (see Figure 5) showed that our Bins have a
479 very similar BGC composition in contrast with reference genomes of the same genus (see Figure 5)
480 revealing that the BGCs are well conserved among multiple strains of the same genus.
481
482 Bin 4 is the MAG with more BGCS with a total of eight BGCS classified as siderophores, terpenes,
483 T3PKS, RiPP-like, NAPAA, ectoine, and NRPS (Figure 5). However, none of the BGCs found in this
484 genome showed a 100% similarity with any known cluster on the MiBiG database therefore, we
485 cannot ensure that any specific metabolite is produced (Table 3). Nevertheless, BGC 22.1 was
486 classified as a RIPP-like (see Table 2) and present a high similarity with Linocin M18 (81%),
487 suggesting that a similar bacteriocin may be produced. Linoncin M18 is a bacteriocin produced by
488 *Brevibacterium linens*; this peptide has been shown to be active against Gram-positive bacteria,
489 particularly *Bacillus subtilis*, *Staphylococcus aureus,* and *Listeria monocytogenes* (Valdés-Stauber et
490 al., 1994). Furthermore, other bacteriocins have been shown to be responsible for the antibiotic
491 activity of different *Brevibacterium* strains; specifically Linencin A (Forquin-Gomez et al., 2014) and
492 Linenscin OC2 (Maisnier-Patin et al., 1995). Linencin A is a 95 KDa peptide that inhibits the growth of
493 only other *B. linus* strains. On the other hand, Linenscin OC2 showed to inhibit the growth of
494 *Arthrobacter*, *Microccus,* and *Corynebacterium.*
495
496 Furthermore, Bin 1 and Bin 2 classified as *Rothia kristinae* showed the presence of NRPS (three
497 BGCs), terpenes (two BGCs), beta lactone (two BGCs), and a T3PKS. Finally, Bin 3 showed six
498 BGCs classified as terpene (one BGCs), beta lactone (one BGCs), NRPS-like (one BGCs), NRPS

(one BGCs), T3PKS (one BGCs), and a siderophore (one BGCs). No BGCs found in the MAGs present a 100% similarity with the ones reported on the MIBIG database, therefore we can not ensure completely that any cluster produces a known secondary metabolite molecule. The low identity of the clusters found in these bins may indicate that novel metabolites are being produced, ensuring the potential of these microorganisms for the bioprospecting of novel antibiotics. Members of the *Rothia* genus have been poorly described as antibiotic-producing bacteria with only a few reports found (Uranga et al., 2020). Particularly, a porcine commensal classified as *Rothia nasimurium* showed the genetic capacity to produce the ionophore antibiotic valinomycin (Roiser et al 2017), but no identification of the compound was performed.

In summary, this work characterizes the bacterial community inhabiting the hair of *Choloepus hoffmani* and *Bradypus variegatus* in a group of animals in captivity. Analysis of the V4 region of the 16S rRNA gene showed that Actinobacteriota, Proteobacteria, and Firmicutes were the most dominant phyla, and *Brevibacterium*, *Janibacter*, *Rubrobacter*, *Brachybacterium*, *Kocuria,* and *Staphylococcus* the most dominant genera across all samples. Additionally, further analysis revealed that even though a significant difference exists between the community inhabiting both sloths' sloths' species, the phylotypes that conform to the core microbiome were mostly shared. Isolation of bacteria revealed that phylotypes closely related to the core microbiome exert an antibiotic activity against a wide variety of pathogens. Furthermore, metagenomics made from the antibiotic-producing strains revealed that multiple BGCs were found in these isolates including siderophores, terpenes, betalactone, Type III polyketide synthases (T3PKS), ribosomally synthesized and post-translationally modified peptides (RiPPs) similar to bacteriocin Linocin M18, non-alpha poly-amino acids (NAPAA) like e-Polylysin, ectoine and nonribosomal peptides (NRPs).

Our results suggest the presence of antibiotic-producing bacteria in the fur of sloths that could modulate the microbial community in that ecosystem. This modulation makes it possible to control the proliferation of potentially pathogenic bacteria for the host or inhibit other competitors (e.g. fungi) in the niche. On the other hand, our results point to sloth fur as a new source of antibiotic-producing bacteria that may open the opportunity for the discovery of new bioactive molecules.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contribution

MC and DRG conceived and designed the experiments; DRG, KVM, RP performed the experiments; DRG, MC and KRJ analyzed the data; MC, JA-A and RP contributed reagents or materials; DRG and MC wrote the paper. All authors reviewed and approved the final version of the manuscript.

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Tables

Table 1. Identity and antimicrobial activity of bacteria isolated from sloths' hair.

Strain	Host	Activity against pathogen														
		SA	EC	PA	BS	CA	SAg	SS	SE	KP	SM	Cy	Co	Fu	Hy	Tr
Rothia koreensis 10C1	*Choloepus*	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rothia kristinae 13C9	*Choloepus*	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
Rothia kristinae 13C8	*Choloepus*	+	-	+	+++	-	-	-	-	-	-	+	-	-	-	-
Rothia sp. 9C1	*Choloepus*	+	-	+	+++	-	-	-	-	-	-	-	-	-	-	-
Rothia kristinae 11C2	*Choloepus*	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-
Rothia kristinae 6B2	*Bradypus*	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-
Rothia kristinae 3B2	*Bradypus*	+	-	+	+++	-	+	-	-	-	-	-	-	-	-	-
Brevibacterium sediminis 11B2	*Bradypus*	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
Rothia kristinae 3B6	*Bradypus*	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-

SA = *S. aureus*; EC = *E. coli*; PA = *P. aeruginosa*; BS = *B. subtilis*; CA = *C. albicans*; Sag = *S. agalatiae*; SS = *S. saprophyticus*; SE = *S. epidermidis*; KP = *K. pneumonie*; SM = *S. marcenses*; Cy = *Cytospora* sp.; Co = *Colleotrichum* sp.; Fu = *Fusarium* sp.; Hy = *Hypocrea* sp; Tr = *Trichoderma* sp.

Table 2. Putative biosynthetic gene clusters in *Rothia koreensis* (Bin 3), *Rothia kristinae* (Bin 1 and Bin 2) and *Brevibacterium* (Bin 4). BGCs were identified using Antismash 5.0 and classified agains the MiBiG database as described in "Experimental procedures".

Bin	Region	Type	Most Similar known BGC	Similarity (%)
Rothia kristinae Bin_1	1	NRPS		
	2	NRPS		
	3	T3PKS		
Rothia kristinae Bin_2	1	Terpene	Carotenoid	35
	2	Betalactone	Microansamycin	7
	3	Betalactone	Microansamycin	7
	4	Terpene	Carotenoid	35
	5	NRPS	Salinichelins	23
Rothia koreensis 3	1	Betalactone	Microansamycin	7
	2	NRPS-like	Kistamicin	12
	3	T3PKS	Endophenazine A1	17
	4	Siderophore	Desferrioxamin B	60
	5	NRPS		
	6	Terpene	Pimaricin	11
Brevibacterium 4	1	Siderophore	Desferrioxamine E	75
	2	Terpene	Carotenoid	71
	3	T3PKS	Endophenazine A1	21
	4	RiPP-like		
	5	NAPAA		
	6	Ectoine	Ectoine	75
	7	NRPS	Pepticinnamin E	10
	8	NAPAA		

933 **Figure legends**

934

935 **Figure 1. Sloths that inhabit in the Sloth Sanctuary of Costa Rica located in Cahuita, Costa**
936 **Rica**. A) *Choloepus hoffmani*, B) *Bradypus variegatus.*

937

938 **Figure 2. SEM images of the hair of *Choloepus hoffmani* and *Bradypus variegatus*.** A)
939 *Choloepus* hair are filament-like structures B) Filaments present a width of 138 ± 13 µm. C) The hair
940 present multiple cavities along its longitude D) These cavities seem to be the niche for the microbial
941 community inhabiting sloth hair. E) The higher abundance of microorganisms in these furrows is
942 probably associated to the lack of mechanical stress they suffer F) Most microorganisms presented a
943 coccoid-shape morphology, also some rod-shaped microorganisms were present. G) *Bradypus* hair
944 is smoother H) It have average width of 261 ± 36 µm. I) It present irregular fissures throughout the
945 entire hair longitude. J) Microorganisms survive in hair surface K) Coccoid and bacilli shaped bacteria
946 are observed across the hair. L) The density of microorganisms is lower probably as a consequence
947 of the hair morphology.

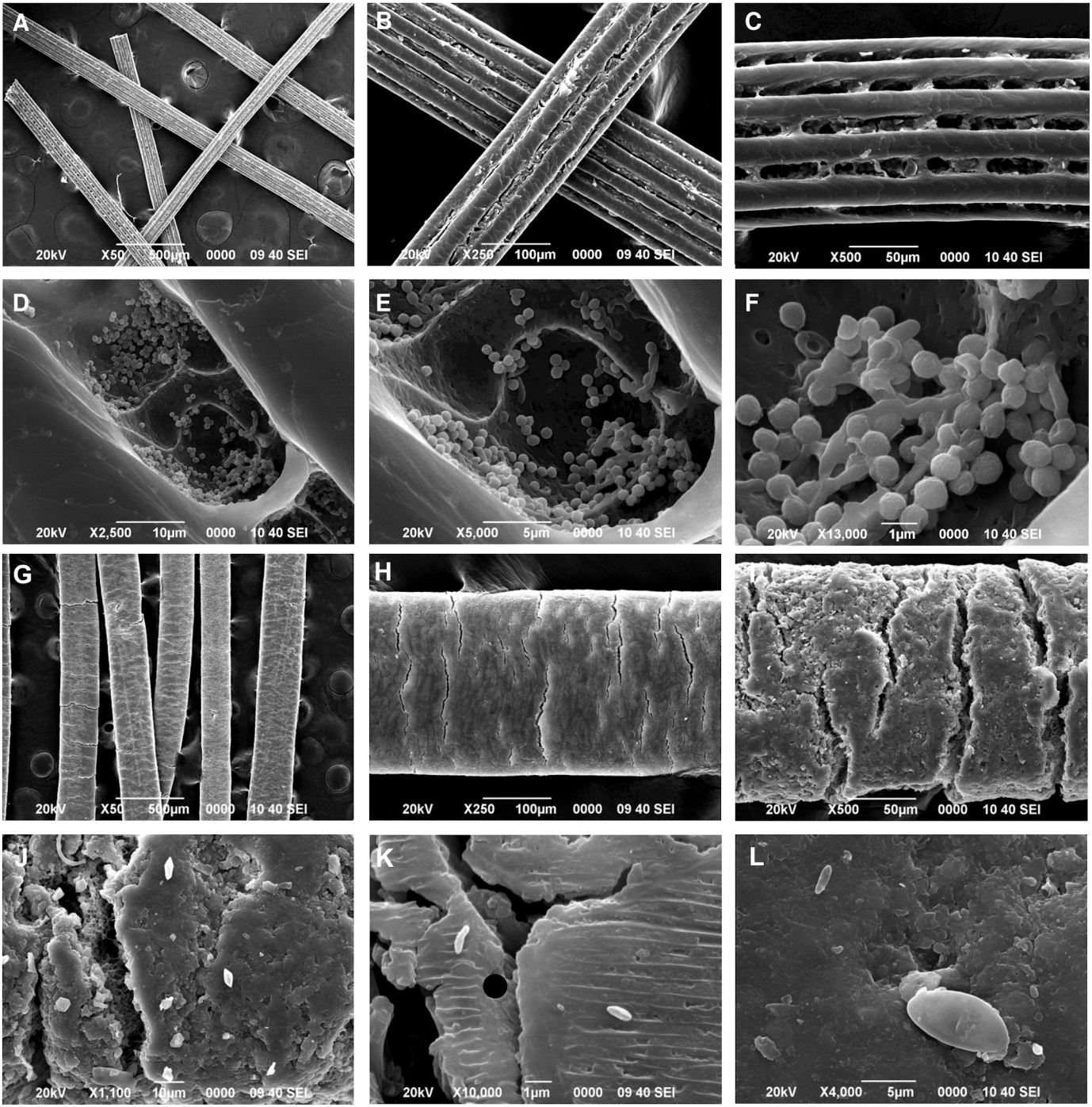
948

949 **Figure 3. Taxonomic composition of prokaryotic community inhabiting the hair of *Bradypus***
950 ***variegatus* and *Choloepus hoffmani*.** Relative abundance of bacterial and archaeal organisms at
951 the A) phylum, B) Order levels. C) Relative percentage of 16S rRNA gene sequences assigned to
952 each ASV (y axis) across the 28 samples analyzed (x axis).The ASV were taxonomically classified
953 using SILVA reference database v138, as described in "Experimental procedures". *Bradypus*
954 samples are identified as B1 to B13 and *Choloepus* samples are identified as C1-C15.

955

956 **Figure 4. Non-metric multidimensional scaling (NMDS) analysis and Venn diagram of the**
957 **prokaryotic communities in the hair of both sloth's species**. A) The NMDS analysis revealed a
958 clustering of communities according to the host is shown. The NMDS and the permanova analyses
959 were performed with Package Vegan v2.5- 6. B) Venn diagram presenting the number of ASV of the
960 core microbiome shared between *Bradypus variegatus* and *Choloepus hoffmani*. Both sloths share
961 almost 50% of the core microbiome. Core microbiome was calculated using the R package
962 microbiome as described in Experimental procedures.
963
964 **Figure 5. Heat map of BGCs types from the MAGs assembly and references genomes**
965 **identified in antiSMASH and BiG-SCAPE.**

A
B


A
20kV X50 500µm 0000 09 40 SEI
B
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C
20kV X500 50µm 0000 10 40 SEI
D
20kV X2,500 10µm 0000 10 40 SEI
E
20kV X5,000 5µm 0000 10 40 SEI
F
20kV X13,000 1µm 0000 10 40 SEI
G
20kV X50 500µm 0000 10 40 SEI
H
20kV X250 100µm 0000 09 40 SEI
I
20kV X500 50µm 0000 10 40 SEI
J
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K
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L
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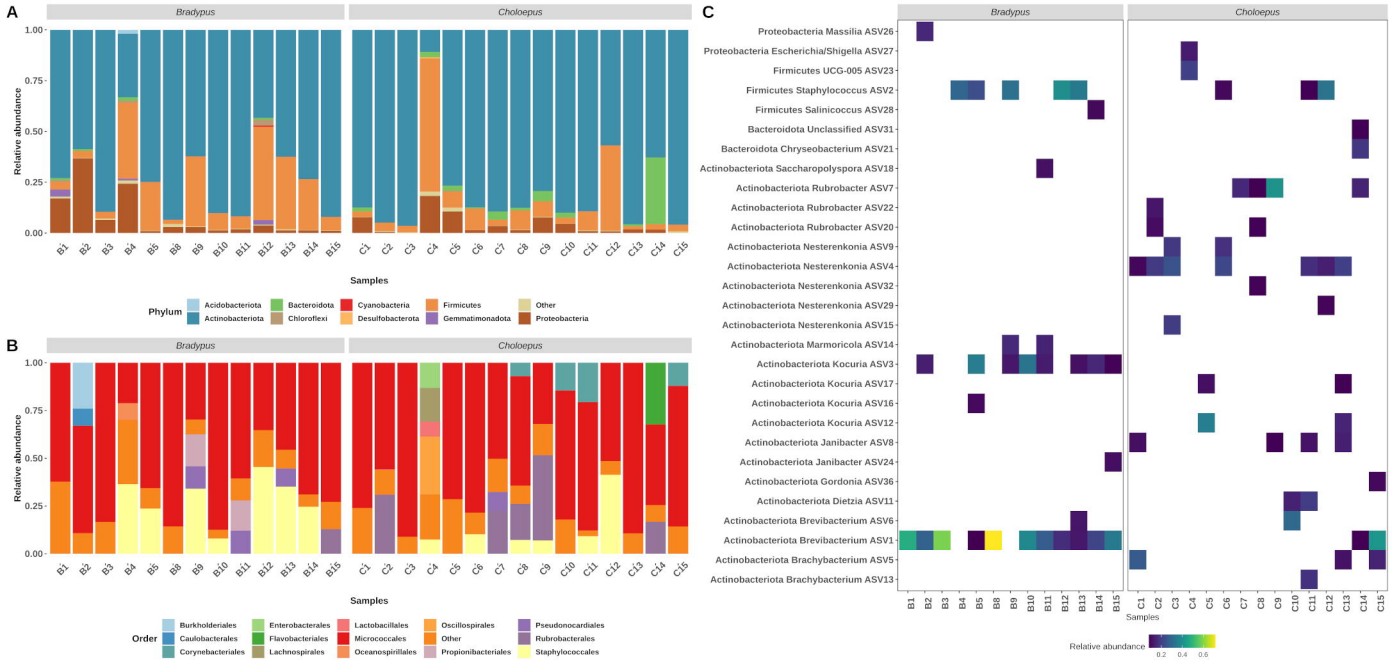
A
Bradypus
Choloepus
Relative abundance
1.00
0.75
0.50
0.25
0.00
B1 B2 B3 B4 B5 B8 B9 B10 B11 B12 B13 B14 B15
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15
Samples
Phylum
Acidobacteriota
Actinobacteriota
Bacteroidota
Chloroflexi
Cyanobacteria
Desulfobacterota
Firmicutes
Gemmatimonadota
Other
Proteobacteria
B
Bradypus
Choloepus
Relative abundance
1.00
0.75
0.50
0.25
0.00
B1 B2 B3 B4 B5 B8 B9 B10 B11 B12 B13 B14 B15
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15
Samples
Order
Burkholderiales
Caulobacterales
Corynebacteriales
Enterobacterales
Flavobacteriales
Lachnospirales
Lactobacillales
Micrococcales
Oceanospirillales
Oscillospirales
Other
Propionibacteriales
Pseudonocardiales
Rubrobacterales
Staphylococcales
C
Bradypus
Choloepus
Proteobacteria Massilia ASV26
Proteobacteria Escherichia/Shigella ASV27
Firmicutes UCG-005 ASV23
Firmicutes Staphylococcus ASV2
Firmicutes Salinicoccus ASV28
Bacteroidota Unclassified ASV31
Bacteroidota Chryseobacterium ASV21
Actinobacteriota Saccharopolyspora ASV18
Actinobacteriota Rubrobacter ASV7
Actinobacteriota Rubrobacter ASV22
Actinobacteriota Rubrobacter ASV20
Actinobacteriota Nesterenkonia ASV9
Actinobacteriota Nesterenkonia ASV4
Actinobacteriota Nesterenkonia ASV32
Actinobacteriota Nesterenkonia ASV29
Actinobacteriota Nesterenkonia ASV15
Actinobacteriota Marmoricola ASV14
Actinobacteriota Kocuria ASV3
Actinobacteriota Kocuria ASV17
Actinobacteriota Kocuria ASV16
Actinobacteriota Kocuria ASV12
Actinobacteriota Janibacter ASV8
Actinobacteriota Janibacter ASV24
Actinobacteriota Gordonia ASV36
Actinobacteriota Dietzia ASV11
Actinobacteriota Brevibacterium ASV6
Actinobacteriota Brevibacterium ASV1
Actinobacteriota Brachybacterium ASV5
Actinobacteriota Brachybacterium ASV13
B1 B2 B3 B4 B5 B8 B9 B10 B11 B12 B13 B14 B15
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15
Samples
Relative abundance
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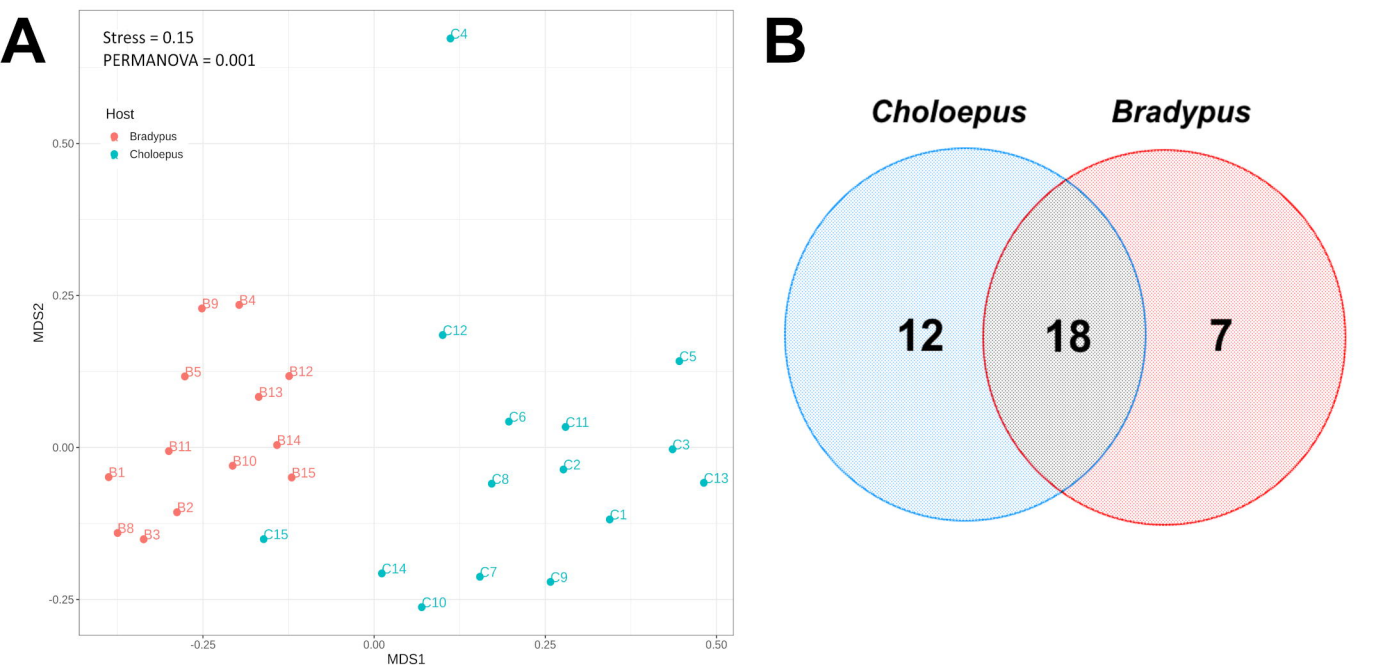
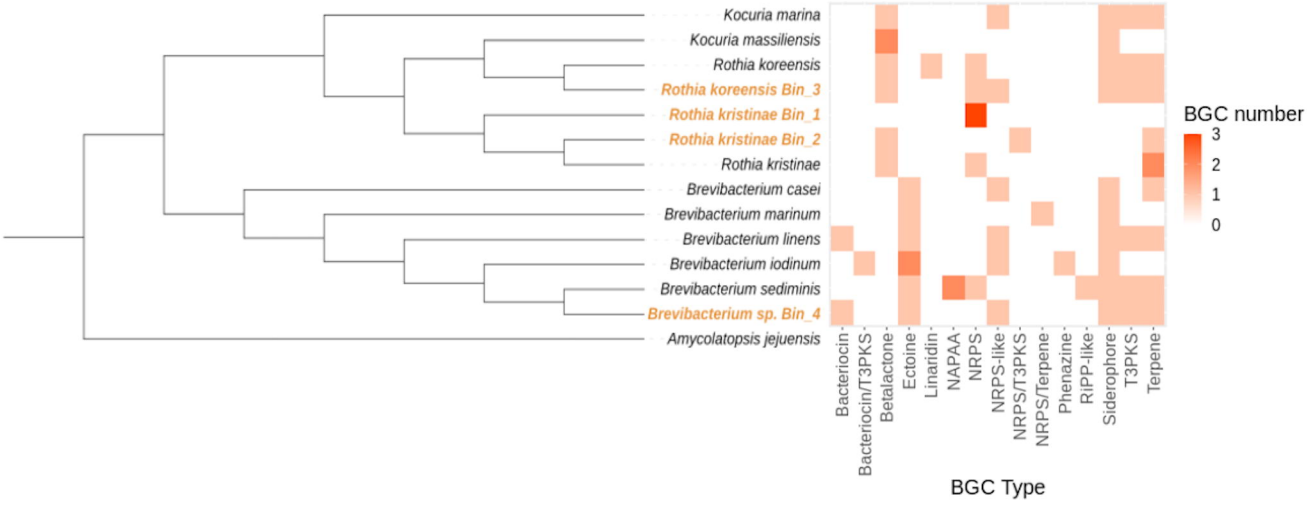
0.4
0.6


A
Stress = 0.15
PERMANOVA = 0.001
Host
Bradypus
Choloepus
MDS2
MDS1
0.50
0.25
0.00
-0.25
-0.25
0.00
0.25
0.50
B9
B4
B5
B12
B13
B11
B14
B10
B1
B15
B2
B8
B3
C4
C12
C5
C6
C11
C3
C2
C8
C13
C1
C15
C14
C7
C9
C10
B
Choloepus
Bradypus
12
18
7


Kocuria marina
Kocuria massiliensis
Rothia koreensis
Rothia koreensis Bin_3
Rothia kristinae Bin_1
Rothia kristinae Bin_2
Rothia kristinae
Brevibacterium casei
Brevibacterium marinum
Brevibacterium linens
Brevibacterium iodinum
Brevibacterium sediminis
Brevibacterium sp. Bin_4
Amycolatopsis jejuensis
Bacteriocin
Bacteriocin/T3PKS
Betalactone
Ectoine
Linaridin
NAPAA
NRPS
NRPS-like
NRPS/T3PKS
NRPS/Terpene
Phenazine
RiPP-like
Siderophore
T3PKS
Terpene
BGC Type
BGC number
3
2
1
0