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CAN GUT MICROBIOTA ANALYSIS BE BENEFICIAL FOR *EX*- AND *IN-SITU* CONSERVATION OF THREATENED ANIMAL SPECIES?

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Abstract

Ex-situ conservation and the *in-situ* conservation of natural habitats are the tools to conserve biodiversity. Habitats and ecosystems have been becoming altered by human activities and a growing number of species requires form of management to ensure their survival. Conservation queries become more complex and urgent. Developing scientifically based and innovative approaches to *ex-situ* conservation is necessary. Recent studies underline importance of gut microbiome in animal health with implications for animal conservation and management. Animal and human studies have demonstrated that environmental factors can impact gut microbiome composition. Within this scenario, the present work focused on species belonging to different taxa, reptiles and mammals: *Aldabrachelys gigantea*, the giant tortoise of the Seychelles islands and *Indri indri,* the greatest leaving lemur of Madagascar. The Seychelles giant tortoise is vulnerable species with declining population, whereas the indri is a critically endangered species that could reach the extinction within 25 years. Both need research to help them to survive. Tortoises live for very long time and to observe how they can afford the environmental changes is very difficult. Indris, instead, are able to survive only in a small area of the Madagascar forest, with a very strong link between the species' survival and the environment. The obtained results underline importance of environmental factors, both *in-situ* and *ex-situ,* for species conservation. Microbiome could help the organisms to respond on a short timescale and cope with, environmental changes. However, species with long generation time might not be able to adapt to fast changes but bacteria with a short generation time can adapt on a shorter timescale allowing the host to cope with fluctuating environment. Gut microbiome plays an important role in an animal's health and has the potential to improve the management of individuals under human care for conservation purposes.

Table of Contents

1 *Ex-situ* Conservation and the microbiota

1.1 The role of the modern zoological garden as place for *ex-situ* conservation

The Convention on Biological Diversity (UN, 1992) is the most comprehensive international agreement ever signed. It looks for conserving the diversity of life on Earth at all levels, from genetic and demographic population to species, from habitat to ecosystem. The Convention also works to maintain the diversity of the life to support systems of the biosphere overall. To achieve the goals of the Convention progress on many fronts is required. Existing knowledge must be used more effectively, and a deeper understanding of human ecology and environmental effects must be gained. The results of research must be communicated to those who can stimulate and shape changes; technologies and practices more environmentally friendly must be applied; international level of technical and financial collaboration is needed. Nowadays, more than in the past, biodiversity conservation is a priority. The origins of efforts to manage global biological diversity can be traced to the United Nations Conference on Human Environment held in Stockholm in 1972. During that conference, biodiversity conservation was defined as a priority. The "Action Plan for the Human Environment: programme development and priorities: report of the Executive Director" adopted in 1973 at the first session of the Governing Council of UNEP pointed out the "conservation of nature, wildlife and genetic resources" as a priority area to work on. In the same decade, the Convention on Wetlands (1971) (UNESCO, 1994), the World Heritage Convention (1972), the Convention on International Trade in Endangered Species (1973), and the Convention on Migratory Species (1979) as well as various regional conventions were adopted, confirming the international importance of conservation.

In June 1992, in Rio de Janeiro, the United Nations Conference on Environment and Development (the `Earth Summit', or UNCED) was held and the Convention on Biological Diversity was opened to sign. The aims of the Convention are "the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilisation of genetic resources" (Article 1). These are developed in the documents from Article 6 to Article 20. The Article 9 of the Convention on Biological Diversity with the title "*Conservation of biodiversity ex-situ*" acknowledges the contribution of ex-situ measures and facilities,

such as gene banks, botanic gardens and zoos, to the conservation and sustainable use of biological diversity (UN, 1972). *Ex-situ* conservation as well the conservation of natural habitats (*in-situ* conservation) are two of the principal tools with which to conserve biodiversity. *Ex-situ* conservation actions are designed to conserve the genetic diversity and populations of species outside their natural habitats. *Ex-situ* conservation measures complement *in-situ* conservation measures and can contribute to ensuring the viability of some threatened wild populations and prevent extinctions (EU, 2015).

Habitats and ecosystems have been becoming altered by human activities and a growing number of species requires form of management of both at individual and population level to ensure their survival. To assess actions addressing the conservation pressures facing a particular species, its conservation plan should consider all options. *Ex-situ* management is one possible option that can contribute to the conservation of threatened species. Indeed, species extinctions have been prevented and for an increasing number of species, conservation and reintroduction programs from *ex-situ* management have been at work. However, the necessity for and appropriateness of an *ex-situ* program have to be adequately considered as part of an integrated conservation strategy. *Ex-situ* programs must be carefully planned and implemented to provide conservation benefits. In addition, as conservation queries have been becoming more complex and urgent, it is necessary to further develop scientifically based and innovative approaches to *ex-situ* conservation (IUCN, 2014).

1.2 *Ex-situ* conservation and the survival of the species

For many threatened vertebrates to live in a controlled environment might be the only choice for survival. The IUCN red list categorizes these species as “Extinct in the wild” but also “Critically Endangered”. There were 73 species considered Extinct in the Wild, and 6,413 were classified as Critically Endangered on the 2019 IUCN Red List of Threatened Species.

However, to understand the effects of living under human care is a key factor for animal management and conservation, including breeding endangered species for release purpose. Environmental changes occurring in different conditions may influence survival ability in the wild. Thus, it is very important to promote *ex-situ* breeding programs for a threatened species and to evaluate the effectiveness and

conservation impact of *ex-situ* breeding efforts (Gippoliti, 2012; Gant et al., 2020). In almost 30 years since the Convention on Biological Diversity was signed, *ex-situ* management has been adopted for several purposes, some well documented (Maunder & Byers, 2005) but others not. To take decisions about the applicability of *ex-situ* management for species conservation goals and targets, it is important to know its contribution to date. Examples of *ex-situ* efforts for conservation of threatened species are within the European Community the LIFE projects with *ex-situ* conservation actions that have targeted mammals, birds, fish, herpetofauna (reptiles and amphibians) invertebrates and plants. These LIFE programmes have been acted in safeguarding endangered flora and fauna through *ex-situ* conservation. LIFE co-funding has been crucial for projects across the EU that have established seed banks or gene banks, set up species centres for *ex-situ* breeding and prepared habitats for the reintroduction of a species or the reinforcement of an existing population. With LIFE's help, these projects have built expert teams, established *ex-situ* conservation protocols and enabled monitoring of reintroduced individuals, all with the goal of improving the conservation status of species. More than 80 LIFE Nature projects have included *ex-situ* conservation measures, targeting a wide range of vulnerable species, ranging from flagship species such as the brown bear, to lesser-known but equally important and endangered species such as the Spanish toothcarp. One of the notable actions of the LIFE projects is the work they have done to raise awareness amongst the general public and key target groups such as farmers and hunters so that reintroduced species are not killed through accidental or deliberate means. This has been especially important for species considered a threat (to people or livestock) such as the Hungarian meadow viper, and, in Spain, the Iberian lynx and bearded vulture (Silva et al., 2019). The examples of LIFE projects should inspire ongoing and future projects that feature *ex-situ* conservation actions with innovative ideas and good practice.

1.3 The microbiota of the animals under human care

The dramatic loss of biodiversity worldwide threats the functioning of ecosystems, their ability to support ecological communities and their resistance to environmental change (Cardinale et al., 2012; Ripple et al., 2016). We should consider an interdisciplinary approach that considers the direct and indirect effects of anthropogenic disturbances on wildlife physiology and health (Campbell, 2005; Cooke

et al., 2013; Wikelski et al., 2006). Innovative ideas and good practice are essential to develop programs and actions to prevent the extinction of species (Cooke et al., 2013; Soule, 1985). Recently research has revealed that host-associated microbiota (archaea, bacteria, fungi and virus) influence animal health and these microbial communities might be altered by anthropogenic activities (Cho & Blaser, 2012; Kohl & Carey, 2016; McFall-Ngai et al., 2013; McKenney et al., 2018; Trevelline et al., 2019). Every metazoan species is a diverse and complex set of microorganisms (viruses, bacteria, fungi and protozoans) known collectively as the microbiota. This multigenomic microcosm can no longer be considered separate from the individual. Thus, it has been proposed that animals represent a complicated biological 'super organism' in which part of the physiological function is derived from microbial activity (Dethlefsen et al. 2007). Therefore, when considering conservation actions, the host-associated microbial diversity should be taken into consideration as it seems to be a serious threat to wildlife populations. Albeit microbiome research has the potentiality to improve conservation outcomes, few efforts have been made to integrate the biodiversity of host-associated microbiota as an important component of wildlife management practices and thus to consider approaches for maintaining microbial diversity to successfully achieve conservation objectives (Trevelline et al, 2019).

As *ex-situ* conservation is one of the two sides of the conservation, and it has been demonstrated that it is necessary for those species that are threatened in their natural environment, every effort should be used to maintain the individual to guarantee the diversity of gut microbiota. The microbiota of the animals in controlled environment should resembled the one of the wild animals because this might affect the fitness with important implications for the conservation and management of species and populations (Trevelline et al., 2019). Controlled environment might alter host-associated microbiota through different mechanisms, such as the adoption of less diverse or compositionally different diets (McKenzie et al. 2017), cohabitation with other species and antibiotic administration (Clayton et al., 2016; Kohl 2014). It has been suggested that there could be differences between microbiomes in individuals between wild and controlled environment and living under different conditions (controlled or wild environment) might impact the species across taxa by diminishing microbiomes. Recent studies show that controlled environment might affect animal microbiomes, reducing symbiotic bacterial diversity and pathogen resistance (Clayton et al., 2016; Li et al., 2017; Lavoie et al., 2018; Mckenzie et al., 2017), significantly

influencing the host health and welfare (Sommer and Backhed, 2013). An altered gut microbiome might have negative effects on an individual health (Lavoie et al., 2018; Mckenzie et al., 2017). As the purposes of the zoos, as recognized institutions for *ex-situ* conservation, are to protect or increase abundance of endangered species aiming at releasing species into the wild, the management of breeding populations should not affect animal microbiomes. It seems that if microbiomes of the individuals being released are altered, this might affect the fitness with subsequently reduction of the probability of successful reintroduction into the wild (Trevelline et al., 2019). Optimizing environmental conditions of species in zoos and botanical gardens could potentially ensure successful management and reintroduction.

Thus, in controlled environment strategies should be used to ameliorate abundance and diversity of microbial communities. However, care must be taken in the selection of candidate microbial reservoirs for wildlife in controlled environment. Hence, whether for one side the *ex-situ* breeding projects seems to be helpful for conservation of biodiversity, on the other hand, it seems that living under human care might affect the diversity, community composition and function of host-associated microbial communities. However, as this field of research is quite new, studies are needed to better understand the influence of different factors on the microbiota in the field of conservation both *in* and *ex situ*.

1.4 Microbiome research for conservation outcomes

Investigating the effects of controlled environment on gut bacterial communities has important implications for animal conservation and management. Host intrinsic factors and environmental factors are associated with the variation in gut bacterial communities (Wang et al., 2018; Knowles et al., 2019). Investigating the causes of these effects might help in minimizing the most influential aspects of controlled environment for diverse microbiome. Natural exposure to environmental microbial reservoirs is thought to be important for maintaining microbial diversity. Controlled environments are different from those of the wild especially because they are away from the land of origin of the species and because the human care management is different from the everyday life in the wild. Changes include diet, range, habitat and climate (Hyde et al., 2016; Mckenzie et al., 2017). Animal and human studies have demonstrated that these environmental factors can strongly impact the gut microbiome

composition (Song et al., 2013; Amato et al., 2015). Results show that diet is one of the major players to gut microbial variation (Muegge et al., 2011; Li et al., 2013; Amato et al., 2015; Carmody et al., 2015, Li et al., 2019). However, it is important to underline that there are numerous factors that could determine the gut microbiome variation and some of them could be naturally occurred such as environmental changes. The microbiome could also play a role to help the organisms to respond on a short timescale and cope with, for example, changes in climate. For species with long generation time, populations might not be able to adapt to fast changes in climate. However, bacteria with a short generation time can adapt on a shorter timescale compared to the host allowing to cope with changes in climate. Such plastic responses can have important implications for persistence of species or populations at risk in a fluctuating environment. Given that the gut microbiome plays an important role in an animal's health and welfare and has the potential to improve the management of individuals under human care.

1.5 General aims

The aims of my PhD project were very ambitious, and they went in the direction to highlight the relevance, possibilities and potential benefits of microbiome research for the field of conservation both *in* and *ex situ*. Seychelles giant tortoises (*Aldabrachelys gigantea*) on one side and the indri (*Indri indri*), the biggest lemur of Madagascar, on the other side are the actors of this project. The two giants belong two different taxa, live on different islands and have different eco-ethological needs. However, both species are herbivores and are threatened and listed in the IUCN Red List with urgent needs for conservation actions. In particular, for both species the *ex-situ* management become crucial for their survival while *in-situ* conservation action should be in place. The study was designed to categorize the gut microbiota of both species to gain a better understanding of how tortoises and indris interact with their environment and determine whether microbiota can act as an indicator of ecological health and welfare both *in* and *ex-situ*. The project has been designed to address a range of general objectives and research aims regarding the microbiota of the two giants. Despite the challenges associated with integrating microbiome research in wildlife management practices, conservation biology and microbiome science have much to offer each other. This PhD project would like to put together these two disciplines to find an effective

and practical way to save species from extinction. Experimental design is one of the most important factors influencing results and interpretations of both microbiome and conservation biology studies. Therefore, conservation biologists and microbiome scientists should communicate each other throughout collaborations. The ideas and techniques from these two fields might produce novel and meaningful results with the potential to increase our scientific understanding while advancing the field of wildlife conservation. Thus, making a link between conservation biologists and microbiome scientists, asking them to collaborate in one project coordinated by a PhD student with zoo experience and in mind the purpose of saving species were the real challenges for this PhD project. Moreover, very important was to build the trust between *in-situ* institution and staff, and *ex-situ* institution and staff to exchange data, knowledge and expertise. Working in different countries, Madagarscar and Seychelles, with different species, lemurs and tortoises, could be examples of how to build relationship. In addition, results from these interdisciplinary projects could highlight the importance of research on gut microbiota for conservation purposes.

2. Gut microbiota of Seychelles giant tortoises both in the wild and controlled environment

Herbivorous reptiles have a hindgut containing high concentrations of bacteria working in an endosymbiotic relationship (Hong et al. 2015). This microbiota produces enzymes needed to ferment carbohydrates such as structural cellulose and hemicellulose that comprises much of their diet (Stevens and Hume 2004). This fermentation produces short chain fatty acids like acetate, propionate, and butyrate (they provide more than 30% of the daily energy that is required for this type of animal), as well as vitamins and amino acids (Mackie et al. 2004; Stevens and Hume 2004). Tortoises are considered keystone species, with ecosystem functions that include burrow construction, which provides habitat for many other vertebrates (Dziadzio and Smith 2016), and seed dispersal due to their herbivore/frugivore diets (Carlson et al. 2003). Maintaining the health of captive individuals, providing all the necessary requirements to sustain them outside their natural habitat remains a challenge. Analyses of the intestinal bacterial microbiota can provide useful information to establish the health status of the hosts and improve strategies for conservation and management of threatened species (Amato 2013; Xie et al. 2016). Intestinal microbiota improves nutrition and energy acquisition, synthesis of essential vitamins, immune system stimulation, and defense from invasive pathogens in the host (O'Hara et al. 2006; Nizza et al. 2014; Pagliari et al. 2015; Shapira 2016). Intestinal bacterial microbiota in vertebrates vary according to differences in their environment and this also might be true for wild versus captive individuals (Xenoulis et al. 2010; Wienemann et al. 2011; Guan et al. 2017). Keeping individuals in under human care may alter the microbial communities compared to wild populations.

Information on the intestinal microbial diversity is not available for *Aldabrachelys gigantea* and for most reptiles. Objective for the study was to characterize and compare the fecal bacterial composition between wild population and population under human care, providing information for decision making for the conservation of this species. Many Seychelles giant tortoises are hosted in zoological and botanical gardens but knowledge should be improved to ameliorate the management of this species with conservation purposes.

2.1 The first paper

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Fecal Microbiota Characterization of Seychelles Giant Tortoises (*Aldabrachelys gigantea*) Living in Both Wild and Controlled Environments

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A microbiome is defined as a complex collection of microorganisms and their genetic material. Studies regarding gut microbiomes of different animals have provided ecological and evolutionary information showing a strong link between health and disease. Very few studies have compared the gut microbiota of animals housed under controlled conditions and those in wild habitats. Little research has been performed on the reptile gut microbiota, and what studies do exist are mainly focused on carnivorous reptiles. The aim of this study was first to describe the overall microbiota structure of Aldabra giant tortoises (*Aldabrachelys gigantea*) and, second, to compare the microbiota of tortoises living under natural conditions and tortoises living in controlled environments, such as zoological and botanical parks, in Italy and in the Seychelles. Seventeen fecal samples were collected from giant tortoises located on Curieuse Island (CI, $n = 8$), at the Botanical Garden (BG, $n = 3$) in Mahé (Seychelles Islands) and at Parco Natura Viva–Garda Zoological Park (PNV, $n = 6$) in Verona (Italy). The V3-V4 region of the 16S rRNA gene was amplified in order to characterize the gut microbiota profile. Overall, the major phyla identified were Bacteroidetes 42%, Firmicutes 32%, and Spirochaetes 9%. A higher microbial diversity (alpha indices) was observed for the BG samples as compared to the PNV samples (Shannon: 5.39 vs. 4.43; InvSimpson: 80.7 vs. 25; Chao1: 584 vs. 377 $p < 0.05$). The results in the present study showed a significant difference in beta diversity between the samples from CI, BG, and PNV ($p = 0.001$), suggesting a different bacterial fecal profile of giant tortoises at the different habitats. This study provided novel insights into the effects of different environmental conditions on the gut microbial communities of giant tortoises. In particular, differences were reported regarding the bacterial gut community structure between tortoises in natural and in controlled environments. These results could help to improve the management of giant tortoises under human care, thus enhancing *ex-situ* conservation efforts far from the species geographic range.

Keywords: *Aldabrachelys gigantea*, giant tortoises, gut microbiota, wild environment, controlled environment

INTRODUCTION

A microbiome is defined as a community of microorganisms (microbiota) and their collective genomes inhabiting a particular environment which includes animals and humans. Hosts benefit from complementing the functions encoded in their own genomes with those of their associated microbiota (Bäckhed et al., 2005).

The symbiotic relationship established between the microbiota and the associated host has been found to be particularly relevant when the gastrointestinal tract was considered (Nicholson et al., 2012). Studies on the gut microbiomes of different animals have provided a wealth of ecological and evolutionary information showing a strong link with health and diseases (Costa et al., 2012). In addition, the influence of the gut microbiome on stress and anxiety as well as on social behavior has been demonstrated (Cryan and Dinan, 2012; Sharon et al., 2016). To date, several studies have focused on the gut microbiota of mammals, especially that of humans, but also of birds, fish and insects, etc. However, little research on this topic has been carried out on reptiles (Scheelings et al., 2020), and has focused mainly on carnivorous species (Arizza et al., 2019; Biagi et al., 2019), whereas herbivorous reptiles are still underrepresented.

Fewer than 2% of reptiles have been described as herbivorous, making herbivore species quite rare within this group (Vitt, 2004). The scarcity of herbivorous reptiles has been related to ectothermy, as their body temperature is too low to allow fermentation (Mackie et al., 2004). Some tortoises, green turtles and lizards have evolved over time becoming herbivorous. They increased their body size or lowered their metabolic rate to increase the food transit time in the gut and adopted several behaviors to maintain a higher body temperature. These adaptations allowed the microbial community to efficiently ferment polysaccharides (King, 1996). To the authors' knowledge, the only studies on the gut microbiota of hindgut-fermenting tortoises have regarded threatened gopher tortoises (*Gopherus polyphemus*) (Gaillard, 2014; Yuan et al., 2015), Bolson tortoises (*Gopherus flavomarginatus*) (García-De la Peña et al., 2019) and Galápagos giant tortoises (*Geochelone nigra*) (Hong et al., 2011). Other studies on herbivorous reptiles have involved green turtles or iguanas (Hong et al., 2011; Ahasan et al., 2018; Campos et al., 2018; Bloodgood et al., 2020; McDermid et al., 2020). No data are available for the Aldabra giant tortoise (*Aldabrachelys gigantea*) gut microbiota, except for the study on gastrointestinal candidiasis in a single Aldabra giant tortoise (Juniantito et al., 2009); this was, however, taken into consideration in the present study. The Aldabra giant tortoise is an endemic species of the Aldabra Atoll, but has also been introduced in many other Seychelles islands (Turnbull et al., 2015). Aldabra giant tortoises have a thick and domed carapace, a long neck, and rough and short legs. They can live solitarily or aggregate in herds, and have a promiscuous mating system (Grubb et al., 1971). They are mainly herbivores and eat mostly grass, leaves, woody plants, herbs and sedges (Grubb et al., 1971; Gerlach et al., 2006). This species is listed as Vulnerable on the International Union for conservation of Nature (IUCN) Red List (IUCN, 1996).

The Aldabra giant tortoise has been considered to be under threat since the late 1800s (Gerlach et al., 2006). Historically, several species of giant tortoises have been present throughout the western Indian Ocean Islands, Madagascar, some of the Mascarene Islands and many of the Seychelles Islands (Gerlach et al., 2013). After human settlement on the islands, the giant tortoise populations declined dramatically as a result of hunting and also of predation of hatchlings by newly introduced predators. Although other wild populations have been reintroduced within and outside the species historic range, only one natural population of Aldabra giant tortoise has currently survived and lives on the Aldabra Atoll (Gerlach et al., 2013). Several tortoises still also exist in captivity on the Seychelles Islands (Mahé, Praslin, and La Digue) and in zoological parks worldwide, and represent a reservoir of this species. However, despite the number of giant tortoises living in zoological institutions, their care and breeding have proven to be difficult. Issues in maintaining healthy populations under human care are still unresolved (Geurts, 1999; Hatt, 2008; Ross, 2019), although correct health care and management of this species in controlled environments are very important for its survival (Jacobson, 1994; Hatt, 2008; Falcón and Hansen, 2018). The composition and diversity of the gut microbiome seem to influence animal behavior and health. Thus, microbiome dissection could be a useful non-invasive method of better understanding the needs of these animals to improve their well-being and welfare. In particular, the exploration of the gut microbial community composition in individuals living both under human care and in the wild, could reveal important features regarding the effect of diet and environment on animal health.

The aims of the present study were to characterize the gut microbiota of the Aldabra giant tortoise and to compare, for the first time, the microbiota of tortoises living under natural conditions, on the Seychelles Islands, with individuals living in controlled environments, in zoological and botanical gardens both in Italy and on the Seychelles Islands, in order to highlight similarities and differences.

The results of this study could provide valuable and practical information regarding the good care, management and health of an *ex-situ* population of Aldabra giant tortoises.

MATERIALS AND METHODS

Target Animals

Thirty-three fecal samples were collected from young and adult giant tortoises. Seventeen were collected from tortoises on Curieuse Island (CI), Seychelles (4°16′56.2″S 55°43′59.7″E), five were collected from tortoises housed at the Botanical Garden (BG) in Victoria at Mahè, Seychelles (−4°37′51.60″S 55°27′4.32″E) and 11 were collected from tortoises housed at Parco Natura Viva – Garda Zoological Park (PNV) in Verona, Italy (45°28′58.3″N 10°47′42.4″E). To identify the sex of each animal, sexual characteristics, such as concavity of plastron and tail length, were used. A tail longer than 20 cm and thicker at the base, and the concave shape of plastron indicated males (Turnbull et al., 2015). When the over-the-curve carapace length

(OCCL) was less than 70 cm and the width of the third dorsal scute was less than 21 cm, the subject was defined as "unknown." Indeed, Aldabra giant tortoises become sexually mature when they reach a size of 70 cm OCCL and have a 3rd dorsal scute of more than 21 cm (Lewis et al., 1991; Beasley et al., 2018). In addition, the number of scales of the tail between the posterior margin of the cloaca and the tail tip also seemed to be a good characteristic for identifying the sex of juvenile animals. As the tail grows, the scales elongate, although new tail scales are not formed. All the juveniles, both males and females, have short tails; it should be noted that female Seychelles tortoises were found to have 8–11 scales while males had 12–14 scales (Gerlach, 2003; Hatt, 2008).

Environment and Housing

The giant Aldabra tortoises on CI roam wild, and they have access to the native island vegetation. They have grass and leaves ad libitum, and endemic fruits and flowers according to the season. They can graze freely near the beach or in the forest. There is also a nursery on the island where the young tortoises, up to 6 years old, are managed by the staff in order to protect them from predators, poaching and also human disturbance. The 2018 annual report of Global Vision International reported the sixth census of the Aldabra giant tortoises on Curieuse Island (Beasley et al., 2018). A total of 122 tortoises were successfully located throughout the island. The majority of the tortoises were located at the Ranger Station, where the study was carried out, with the others dispersed throughout the island (Sanchez et al., 2015). In the nursery, at the time of the study there were 74 young tortoises of different ages; four juveniles of approximately 5 years of age were kept in a separated area of the nursery. The diet of these young tortoises is prepared by the staff of the Seychelles National Parks Authority (SNPA) on Curieuse Island by collecting all the young leaves from the island and, once a week, commercial fruits are added to the diet. Aldabra giant tortoises at the BG are housed in a 1000 m^2 enclosure on different levels, containing rocks, sandy areas, water and muddy pools. More than 30 adult giant tortoises coming from private owners are housed at the BG where they are fed with fresh branches and leaves endemic to the Seychelles. Some fruit is also available. In addition, banana leaves are prepared by the staff and given to the public several times per day as visitors are allowed to directly feed the tortoises. The giant tortoises at PNV are housed in an enclosure consisting of an indoor and an outdoor area. Both areas are divided into two sections, one housing adult tortoises (two males and one female of over 80 years of age) and one housing the youngest tortoises (13 years old). The tortoises have constant access to their indoor area which contains both ultraviolet and heat lamps, a pool area and sand. The tortoises are housed in the indoor area overnight, in cold weather (<18°C) and during the winter for roughly 5 months. For the rest of the year, they have access to the outside area (measuring 1040 m^2). Aldabra giant tortoises at the PNV are fed regularly (4 days per week) with a mixture of leafy greens and vegetables. Once a week, they are fed with seasonal fruit as well as hay. Supplements, such as calcium, are provided. The tortoises only have access to grass and the opportunity to graze over the spring and summer months.

Agreement in Compliance With the Nagoya Protocol on Access and Benefit Sharing of Genetic Resources

Sampling was carried out according to the Nagoya Protocol in agreement with the European Commission Guidance document regarding the scope of application and core obligations (EC, 2016). This protocol requires that an agreement has to be in place between the country providing the genetic resource and the country involved in the research for the exchange of the genetic material; this is mandatory in the countries which ratified the Convention of Biological Diversity (CBD, 1992). Thus, in December 2018, an agreement was signed between Parco Natura Viva, an Italian zoological park, (recipient) and the Ministry of Environment, Energy and Climate Change of the Seychelles (Supplier) to collect and utilize samples for scientific purposes only. For the same purpose, an agreement was also signed between Parco Natura Viva and the Seychelles National Parks Authority (SNPA), the body responsible for all the marine and terrestrial national parks of the Seychelles; Curieuse Island is one of the marine national parks.

Fecal Sample Collection and Bacterial DNA Extraction

The fecal samples were obtained in the early morning, in the late morning and in the early afternoon following the activity patterns of the tortoises. Approximately 5 g of fecal sample were collected into screw-cap tubes with an integrated plastic shovel-like tool attached to the cap, containing 10 ml of RNAlater (Thermo Fisher Scientific, Waltham, MA, United States). Although field conditions did not allow precise measurement of the amount of feces collected, any resultant error could be assumed to be randomly distributed. Fresh feces were collected from each tortoise which was recognized by means of tags or by the particular morphology of the carapace. Disposable sterile gloves were worn when collecting the samples in order to avoid human contamination. In particular, the amount of stool was taken from the middle of each large, fresh and intact piece of feces to avoid soil contamination. The small plastic shovel-like tool attached to the cap of the screw cap tubes was then used to scoop up the fecal samples. Each container was sealed immediately after feces collection in order to avoid cross contamination between the samples. All samples were maintained in a portable cooler with ice packs or in a refrigerator before arrival at the lab.

Total DNA extraction from the fecal samples was carried out using a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with a modified protocol, as previously shown (Yu and Morrison, 2004; Michelini et al., 2015). In the first step, 1.5 mL of the mixture in RNAlater was first centrifuged for 15 min at 3000 × *g*, and the supernatant was discharged. At the end of the purification step, the DNA was quantified using NanoDrop, and was stored at −20°C until library preparation.

PCR Amplification and Sequencing [Next-Generation Sequencing (NGS)]

The V3-V4 regions of the 16S rRNA gene were sequenced using the Illumina MiSeq platform. The amplification of good

quality DNA was obtained from 17 out of the 33 samples collected. In particular, eight samples were from tortoises on CI (CI, n = 8), three from animals at BG (BG, n = 3) and six were from tortoises at PNV (PNV, n = 6) (**Table 1**). Gene amplicons were produced using the primers Pro341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNBGCASCAG-3′ and Pro805R:5′-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014), using Platinum^{TM} Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy). The PCR reaction conditions for amplification of DNA were as follows: initial denaturation at 94°C for 1′, followed by 25 cycles of denaturation at 94°C for 30″, annealing at 55°C for 30″ and extension 65°C for 45″, ending with 1 cycle at 68°C for 7′. The libraries were prepared using the standard protocol for MiSeq Reagent Kit v3 and were sequenced on the MiSeq platform (Illumina Inc., San Diego, CA, United States). The raw sequences were processed using the DADA2 pipeline, and the Silva (release 132) database was used as reference for taxonomy assignment. For the DADA2 pipeline, primers were removed from the raw sequences, based on the average quality score, forward and reverse reads were trimmed at position 290 and 250. All other DADA2 parameters were left with their default settings.

The raw reads obtained are publicly available at the European Nucleotide Archive (ENA) under the accession number PRJEB37279.

Statistical Analysis

The statistical analysis was carried out in an R v3.6 environment (R Core Team, 2019) using the PhyloSeq (McMurdie and Holmes, 2013), Vegan (Dixon, 2003) and lme4 bate (Bates et al., 2015) packages. The alpha diversity indices (Shannon, InvSimpson and Chao1) were calculated, and normality was tested using the Shapiro–Wilk test. Differences were analyzed using an analysis of variance (ANOVA) model considering location (CI, BG, PNV), sex (M or F) and age (categorized as follows: "1" < 20 years, 20 < "2" < 70 years, "3" > 70 years) as fixed factors; sex and age were separated based on the entire study population. When the assumption of normality was not met, the non-parametric Kruskal–Wallis rank sum test together with Dunn's test as *post-hoc* were used. For the beta diversity, a Non-metric Multi-dimensional Scaling (NMDS) plot using Bray-Curtis distance matrix was created. The effect of location, sex and age was tested using the Adonis function with 999 permutations, and the pairwise comparison was carried out using the pairwise Adonis function (Martinez Arbizu, 2020). Prior to the Adonis test, the homogeneity of dispersion among the different locations and among age was tested using the betadisper function. Variables were removed from the model when not significant. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was then used to identify taxa associated with the different locations; LEfSe aids in implementing different statistical tests involving first, a non-parametric factorial Kruskal–Wallis rank sum test, second, a pairwise test using the unpaired Wilcoxon sum-rank test and, finally, linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant amplicon sequence variant (ASV).

The results were considered significant when p was < 0.05, and tendencies were $0.05 < p < 0.10$; a false discovery rate (FDR) < 0.1 and an LDA score cutoff of two were used in order to distinguish the differential abundant taxa.

TABLE 1 | Sampling and features of giant tortoises.

Fecal Sample ID	Tortoise name	Location[1]	Country	Age	Sex
BLB	Bulbo	PNV	Italy	>100	M
PRS	Priscilla	PNV	Italy	>80	F
T32	32	PNV	Italy	13	F
T33	33	PNV	Italy	13	F
T52	52	PNV	Italy	13	F
T53	53	PNV	Italy	13	F
S2	2-NN	CI	Seychelles	25–30	M
S3	3-018	CI	Seychelles	70–80	M
S4	4-C100	CI	Seychelles	80–90	M
S7	7-NN	CI	Seychelles	35–40	F
S10	10-NN	CI	Seychelles	30–35	F
S11	11-NN	CI	Seychelles	20–25	F
S16	16-NN	CI	Seychelles	5	NA
S17	17-NN	CI	Seychelles	5	NA
S18	18-1	BG	Seychelles	60–70	M
S19	19-2	BG	Seychelles	40–50	F
S21	21-4	BG	Seychelles	100	M

[1] *PNV, Parco Natura Viva; CI, Curieuse Island; BG, Botanical Garden.*

RESULTS

Sequencing Output and Analysis

Seventeen out of the thirty-three samples were analyzed since, for the remaining sixteen samples, the DNA extraction did not provide DNA in a sufficient quantity and quality to ensure the amplification of the V3-V4 region. This was probably due to the high amount of vegetal material in the fecal samples.

A total of 708,973 good quality reads were filtered from the 1,017,914 raw reads obtained from the 17 fecal samples (**Supplementary Table S1**). The relative rarefaction curves are reported in **Figure 1**. The tendency to a plateau for the curves of each sample suggested that the sequencing depth was sufficient for describing the variability within the microbial communities analyzed. The DADA2 pipeline identified a total of 3098 unique ASVs from which a total of 25 different phyla (42% Bacteroidetes, 32% Firmicutes, 9% Spirochaetes, 4% Proteobacteria, 3% Tenericutes), 52 classes (Bacteroidia 38%, Clostridia 30%, Spirochaetia 7%, Gammaproteobacteria 4%), 167 families (14% Ruminococcaceae, 14% Rikenellaceae, 8% Spirochaetaceae, 7% vadinHA21, 5% Lachnospiraceae) and 310 genera (7% *Treponema*, 6% Rikenellaceae_RC9_gut_group, 4% DMER64, 3% Ruminococcaceae_UCG_010, 2% *Paludibacter*) were identified among the samples. The relative abundance of the 10 most abundant taxa, at the phylum, class, family and genus levels, is shown in **Figure 2**. Relative abundances of taxa

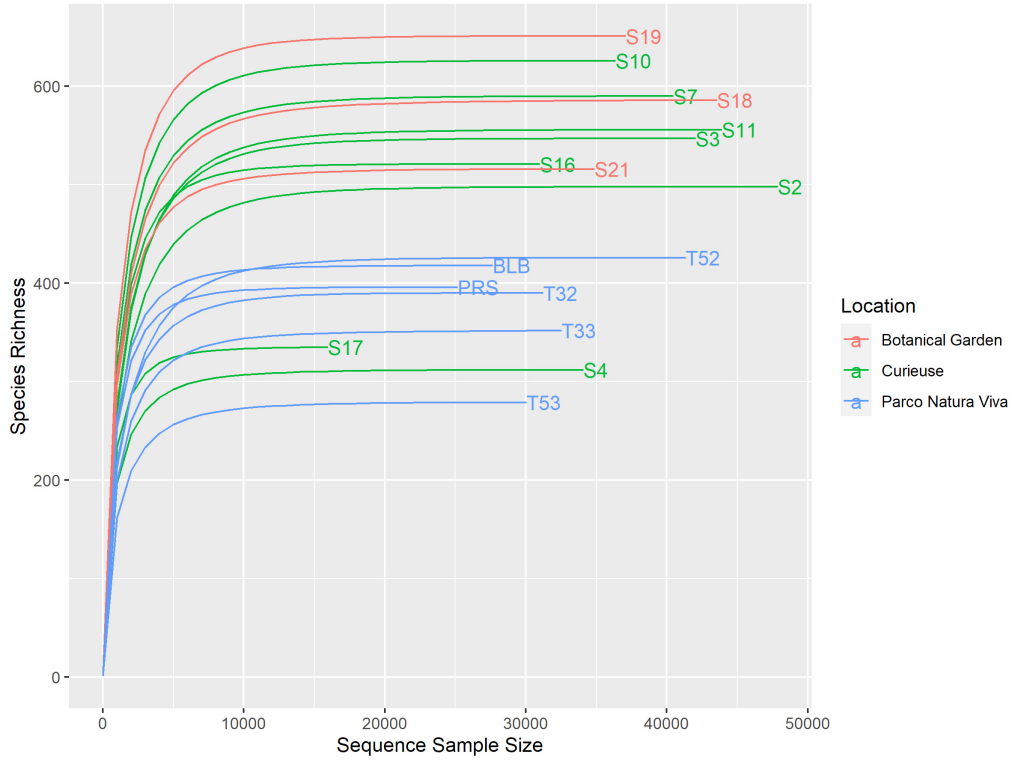

FIGURE 1 | Rarefaction curves of the samples. Different colors have been used for the samples regarding the different conditions.

for each taxonomic rank can be found in the **Supplementary Material** (**Supplementary Table S2**).

Results for alpha diversity, defined as the average species diversity within samples, are reported in **Supplementary Table S3** and **Figure 3**. Location significantly influenced the Chao1 [$F(2) = 5.0$, SS = 62422, $p < 0.05$], the Shannon [$F(2) = 5.2$, SS = 1.9, $p < 0.05$] and the InvSimpson [$H(2) = 7.06$, $p < 0.05$] diversity indices. A significantly higher diversity was observed in the BG samples as compared to the PNV samples for all the indices used ($p < 0.05$), although the results could have been biased by the low number of samples in the BG group. Furthermore, the samples from CI tended to have a higher Shannon index value as compared to the PNV samples ($p = 0.07$); there were no differences between BG and CI, and sex and age did not influence the alpha diversity indices.

Regarding beta diversity, **Figure 4** shows the NMDS plot using the Bray-Curtis distance matrix; the samples from PNV and BG separate into two distinct clusters whereas the samples from CI tend to be more spread out. The Adonis test showed that the microbiological composition of the samples was significantly influenced by location ($p = 0.001$, $R^2 = 0.30$), and also tended to be influenced by age ($p = 0.07$, $R^2 = 0.07$) while no significant effect was observed for sex. Each pairwise comparison regarding the location factor was significant (CI vs. BG: $F = 1.70$, $R^2 = 0.16$, p.adj = 0.03; CI vs. PNV: $F = 4.04$, $R^2 = 0.25$, p.adj = 0.002; BG vs. PNV: $F = 3.53$, $R^2 = 0.33$, p.adj = 0.02). The homogeneity of dispersion between the locations was significantly different ($p = 0.001$), indicating that the results from the Adonis test regarding location could have been influenced by the different dispersion of microbial composition within the samples in the different locations. The samples from the CI group were the most heterogeneous (**Figure 5**). In addition, the homogeneity of dispersion between age categories was not significant, thereby confirming the results of the Adonis test.

In order to identify specific taxa, the abundance of which was influenced by the different locations, the biomarker discovery approach called LEfSe (linear discriminate analysis coupled with effect size measurement) was applied. The LEfSe approach identified 34 bacterial taxa which were differentially abundant among the three groups (**Figure 5**). The tortoises from PNV were characterized by a greater abundance of vadinHA21, Marinilabiliaceae and Pedosphaeraceae at the family level (FDR < 0.1), a greater abundance of *Parabacteroides* genus (FDR = 0.045) and a greater abundance of the specific bacterial species *Campylobacter iguonorum* (FDR = 0.051). The tortoises from the BG were represented by a greater abundance of Clostridiales Family_XIII and the Dysgonomonadaceae families (FDR < 0.1), and also a significantly higher level of bacteria from the genus *Anaerocella* (FDR = 0.045). The tortoises from CI had higher levels of Peptostreptococcaceae and Clostridiaceae_1 (FDR < 0.1). At the ASV level, 15 ASVs were enriched in the BG samples, 8 ASVs in the CI samples and only 1 ASV in the PNV samples; the corresponding ASV classification is reported in the **Supplementary Table S4**.

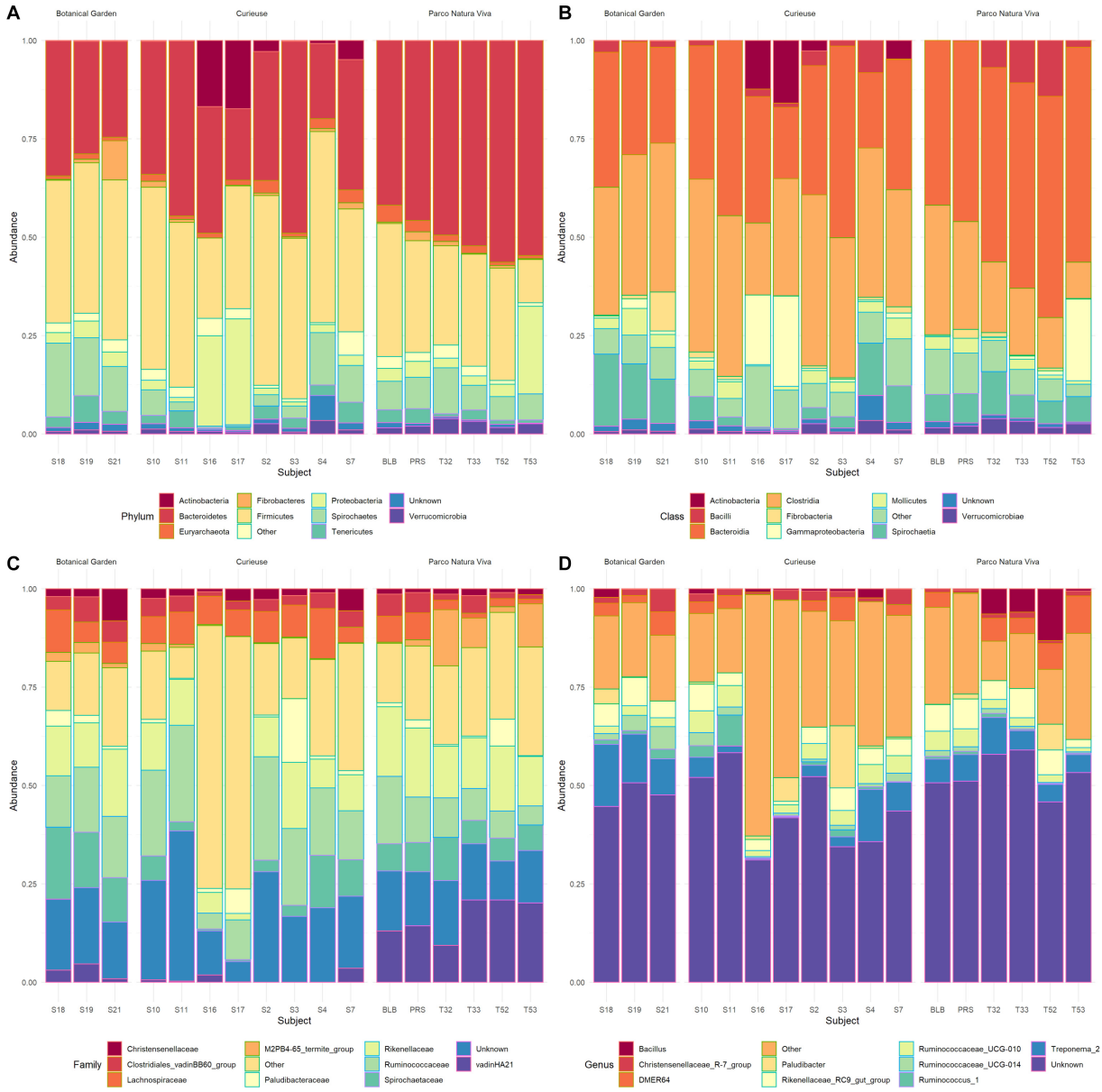

FIGURE 2 | Bar plots representing the percentage abundance of the top 10 Phyla **(A)**, top 10 Classes **(B)**, top 10 Families **(C)**, and top 10 Genera **(D)**. The samples are grouped based on location.

DISCUSSION

Comparisons of the gut microbiota between wild animals and those in controlled environments are very scarce, although they can be important in evaluating whether the goals of breeding programs for endangered species are being properly met. In particular, comparing the microbial composition of the fecal microbiota between wild animals and those in controlled environments could provide information regarding gut microbial diversity. Since diet is one of the main factors modulating the microbial profile, data from this comparison can be useful in improving and personalizing the feeding regimes of animals in a controlled environment. An optimal microbial gut population resulting from diets resembling those of wild tortoises would enhance both the care and well-being of the tortoises as well as the breeding programs of those species under human care.

To the authors' knowledge, little research has been carried out on the gut microbiome of herbivorous turtles and tortoises, and has been focused on threatened gopher tortoises (*Geopherus polyphemus*) (Gaillard, 2014), Galápagos giant tortoises (*Geochelonia nigra*) (Hong et al., 2011), Bolson tortoise (*Gopherus flavomarginatus*) (García-De la Peña et al., 2019) and green turtles (*Chelonia mydas*) (Ahasan et al., 2018; Campos et al., 2018; Bloodgood et al., 2020; McDermid et al., 2020)

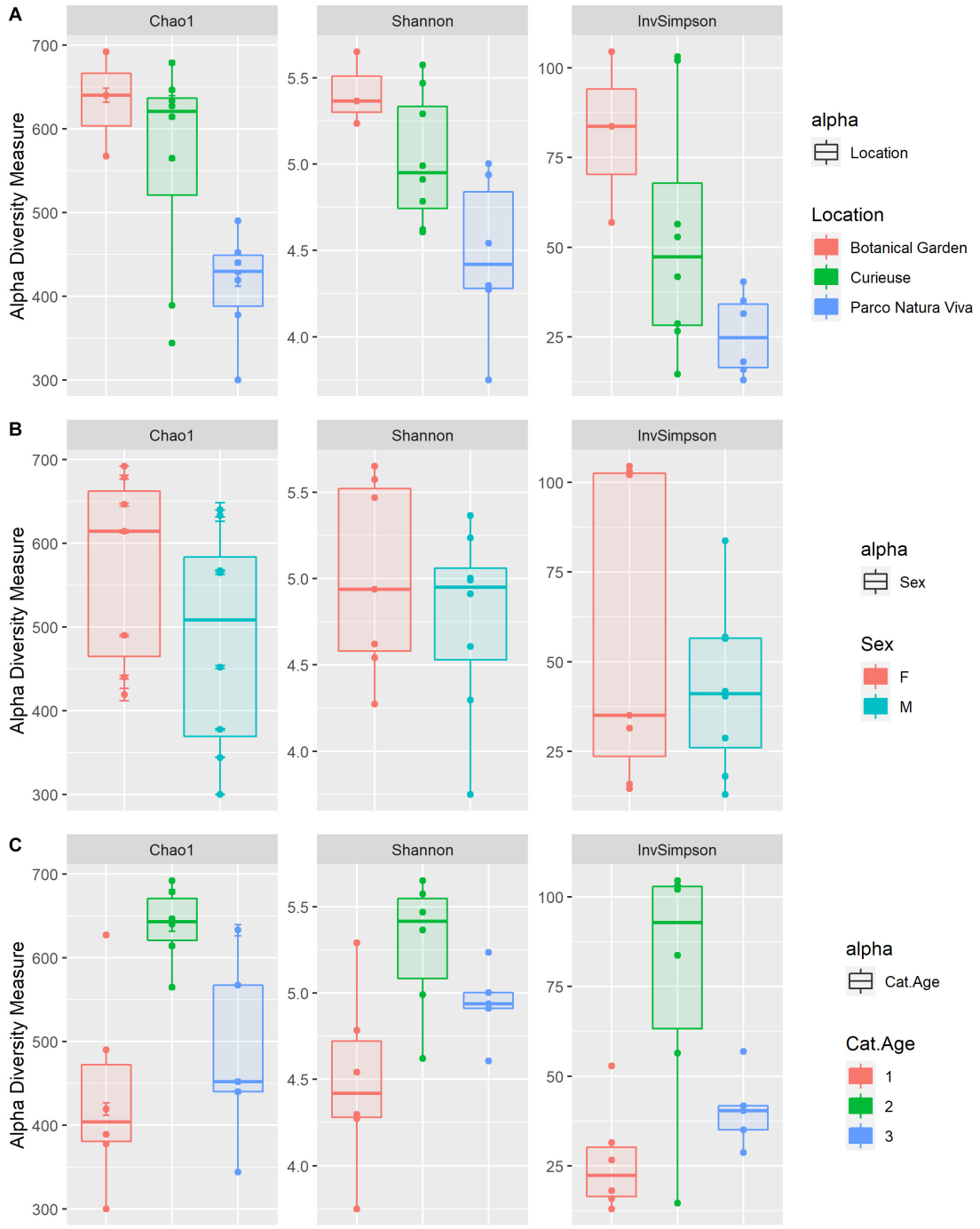

FIGURE 3 | Box plots of the alpha diversity indices (Chao1, Shannon, InvSimpson) estimated for the different groups: Location **(A)**, Sex **(B)**, and Age categories ("1" < 20 years, 20 < "2" < 70 years, "3" > 70 years) **(C)**.

whereas no studies have characterized the gut microbiome of the Aldabra giant tortoise. Analysis of the fecal bacterial community composition revealed that the phylum Bacteroidetes represented the major part of the microbiota, accounting for 42% of the total, as previously reported (Thomas et al., 2011). One of the main functions of Bacteroidetes is the degradation of complex polysaccharides, such as plant cell wall compounds (e.g., cellulose, pectin and xylan). Within the phylum Bacteroidetes, the most represented families were Rikenellaceae and Vadin_HA 21 (32 and 16%, respectively).

Firmicutes was the second most abundant bacterial phylum (32%). Within this phylum, the most represented families were

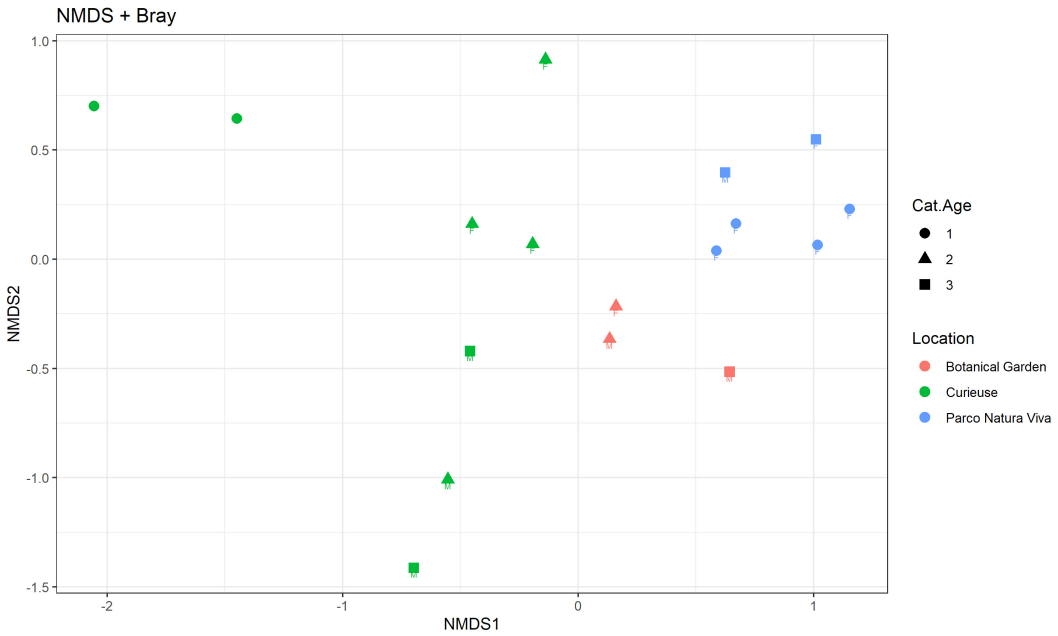

FIGURE 4 | Non-metric multidimensional scaling (NMDS) plot using the Bray-Curtis dissimilarity matrix. The samples are colored based on the location, shaped based on the age category ("1" < 20 years, 20 < "2" < 70 years, "3" > 70 years) and labeled based on sex.

Ruminococcaceae (42%) and Lachnospiraceae (16%) which have a well-known potential for degrading complex carbohydrates of plant origin. These findings are in line with those of studies on hindgut-fermenting tortoises (Yuan et al., 2015). Terrestrial herbivores are characterized by a greater abundance of Ruminococcaceae. Instead, marine herbivores, such as marine iguanas (Hong et al., 2011) and green turtles (Campos et al., 2018), are characterized by a greater abundance of Lanchonospiraceae. This difference could be related to a diet rich in polysaccharides (such as that of terrestrial herbivores) which provides a different fermenting substrate for the microbiota.

The findings of the current study revealed that Bacteroidetes and Firmicutes represented the two major phyla in Aldabra giant tortoises, as reported in studies on other herbivorous tortoises and herbivorous reptiles in general (Hong et al., 2011; Ahasan et al., 2018; Campos et al., 2018; Bloodgood et al., 2020; McDermid et al., 2020; Montoya-Ciriaco et al., 2020). However, the Bacteroidetes/Firmicutes ratio observed in the present study regarding giant tortoises was not in line with that reported by other authors who focused on herbivorous reptiles, specifically tortoises (Hong et al., 2011; Gaillard, 2014). In contrast, Yuan et al. (2015) confirmed the results of the present study, reporting a higher prevalence of Bacteroidetes over Firmicutes in gopher tortoises. Studies on carnivorous reptiles of the Testudines order, such as carnivorous sea turtles, showed that Firmicutes and Bacteroidetes were also the major phyla of their gut microbiota, even though differences in the ratio were present (Abdelrhman et al., 2016; Arizza et al., 2019), presumably due to different diets, climates, habitats or phylogenetic distances (Pluske et al., 1997; Hasan and Yang, 2019; Scheelings et al., 2020).

Other less represented phyla reported in the current study were Spirochaetes (9%) and Proteobacteria (4%). Spirochaetes were mostly composed of *Treponema* (82.7%), as has also been reported by Yuan et al. (2015). Even though Spirochaetes do not have cellulolytic activity, some species have been shown to facilitate the digestion of cellulose by the co-occurring bacteria (Kudo et al., 1987) and to ferment the polymers commonly present in plant materials (Paster and Canale-Parola, 1982). Similar values of Proteobacteria were also found in gopher tortoises (Gaillard, 2014).

Some recent studies have reported differences in microbiota abundance and composition in wild animals as compared to animals in captivity (Cabana et al., 2019; García-De la Peña et al., 2019; Gibson et al., 2019; Tong et al., 2019). In the present study, the alpha diversity index was significantly higher in the BG giant tortoises than in the PNV giant tortoises. The Simpson index was higher in the CI giant tortoises than the PNV tortoises whereas no differences between the BG and the CI giant tortoises were observed. However, caution is needed when interpreting the results regarding the BG samples due to the low number of tortoises which were sampled.

The Adonis test on the Bray-Curtis dissimilarity matrix confirmed that the major factor shaping the microbial composition was represented by the environment. The CI samples had a higher dispersion as compared to the BG and PNV samples. These differences could be explained by differences in the diet. Tortoises in controlled environments (BG and PNV) tended to follow the same diet whereas wild tortoises tended to feed on a wide range of foodstuffs conditioned by seasons. However, these findings could have been biased by different variances between the groups, as suggested by the significant

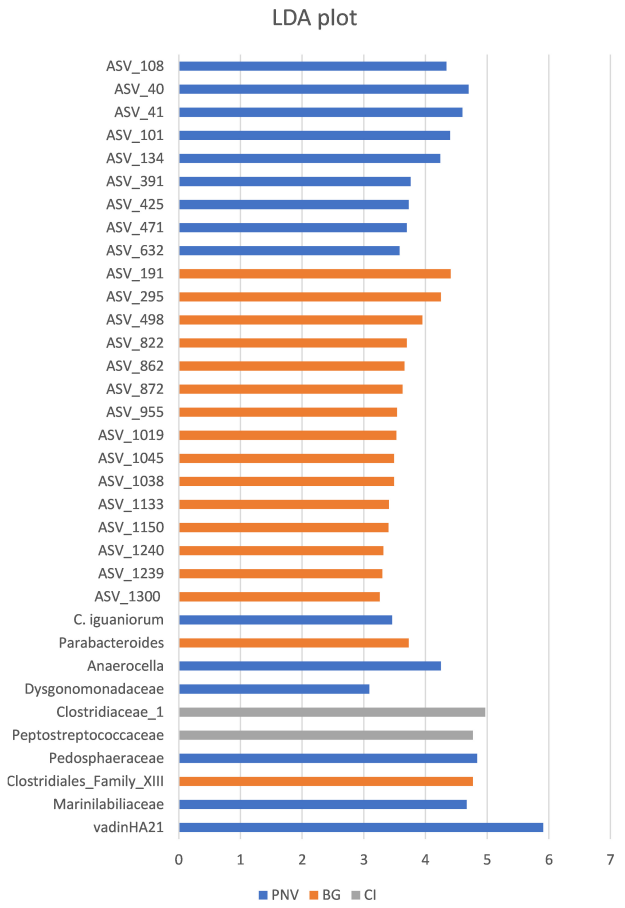

FIGURE 5 | Linear discriminant analysis (LDA) plot, showing the effect size values of different significant taxa among the locations.

beta dispersion analysis ($p < 0.01$). Additional future studies should focus on the effect of location on the beta diversity of fecal microbiota in Aldabra giant tortoises.

The study also focused on the differences in the microbial community composition of the fecal samples from the tortoises in the different locations. The CI Aldabra giant tortoises showed a greater abundance of Peptostreptococcaceae and Clostridiaceae_1. As detailed by Wüst et al. (2011), Peptostreptococcaceae are closely related to Clostridiaceae which are obligate anerobic bacteria capable of consuming plant-derived saccharides. Peptostreptococcaceae are usually considered commensal bacteria, and their presence increases in the gut microbiota of healthy animals (Leng et al., 2016). The phylum Actinobacteria was the most abundant (note that *Bifidobacterium* belongs to this phylum) in the CI tortoises, even if no significant differences were observed. In the CI tortoises, they accounted for 2.97% of the total bacterial phyla whereas, in the PNV and the BG tortoise fecal samples, they represented only 0.02 and 0.1%, respectively. Differences between the CI Aldabra giant tortoises on the one hand, and between BG and PNV tortoises on the other hand, seemed to agree with the results of a recent study by Cabana et al. (2019) in which a greater abundance of *Bifidobacterium* in wild versus captive Javan slow loris was observed. In addition, the highest abundance value of the Actinobacteria phylum (17% of the total phyla) was observed in the two youngest subjects (S16 and S17: 5 years old) in the CI group. Interestingly, this result was in agreement with studies on humans in which Actinobacteria were mainly related to the gut microbial community composition of infants (Schwartz et al., 2012). As reported in recent human studies (Senghor et al., 2018), gut microbiota composition differed not only in different locations but also in different groups within the same area, suggesting that the influence of diet on gut-microbiota composition was as important and relevant as the individual geographical provenance.

The present results showed similarities between the microbiota of tortoises under controlled conditions despite their geographic localization whereas differences emerged between wild tortoises and those living under controlled conditions, even in the same geographical area. These findings might suggest that the composition of the gut microbiota could also be influenced by the environmental conditions under which an animal lives. Of the diverse environmental components, diet could represent one of the most important factors responsible for driving the microbial shift reported in the study groups. In fact, it has been well recognized that, among the factors capable of influencing the microbial profile, diet seemed to be one of the most important, giving reproducible and rapid results (David et al., 2014).

Nutrition is an important component regarding the care of species in a controlled environment. A correct diet plays an important role as a preventive health measure, also encouraging successful mating behaviors (Jacobson, 1994; Hatt, 2008). Providing a correct diet for reptiles, and also for tortoises, is essential for the correct development of the animals. Even though several zoological and botanical gardens maintain Aldabra giant tortoises, knowledge regarding their nutrient requirements is still limited (Ross, 2019).

Overall, the present study suggested that different environmental conditions could drive a shift in the microbial profile of *A. gigantea*. This could be mainly attributed to different diets. This study improved the current knowledge regarding the fecal microbial profile of *A. gigantea*, and provided novel insights into the influence of different environmental conditions on the microbial communities of the gut microbiota of this species. In particular, information regarding the differences in the bacterial gut community structure between tortoises in natural and in controlled environments can be of great value in improving the management and well-being of *ex-situ* Aldabra giant tortoises. Additional studies are needed to better understand this topic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB37279.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because The present work used only fecal samples of tortoises and this does not require the ethical committee approval. Fresh feces were collected by the Animal Care Staff (keepers) during their routine cleaning of the enclosure or directly from soil without manipulating the animals.

AUTHOR CONTRIBUTIONS

PT, CSa, CSp, SR, M-MM, PM, and CZ conceived and designed the experiments. CSa and CSp collected the fecal samples. CSa, FC, DL, and MM carried out the experiments. FC, DL, and AC analyzed the data. CSa, CSp, PT, PM, and MM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.569249/full#supplementary-material

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Disentangling Fecal Microbiota Characterization of Seychelles Giant Tortoises (*Aldabrachelys gigantea*) Living in Both Wild and Controlled Environments

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Contents

Table S1. Number of reads that survived in every step of the bioinformatic analysis.

Sequence ID	Subject	Location	input	filtered	denoisedF	denoisedR	merged	nonchim
359151F354786	S2	Curieuse	55855	53989	52317	52083	48572	48240
359152F354787	S3	Curieuse	50194	48806	47316	47073	42894	42514
359153F354788	S4	Curieuse	39643	38050	37018	36833	34540	34314
359154F354789	S7	Curieuse	48087	46530	44988	44676	41076	40761
359155F354790	S10	Curieuse	44174	42859	40986	41300	36929	36685
359156F354791	S11	Curieuse	51136	49674	48463	48137	44600	44237
359157F354792	S16	Curieuse	41590	39976	37333	37226	32068	31665
359158F354793	S17	Curieuse	21897	21127	19439	19390	16437	16332
359159F354794	S18	Botanical Garden	50787	49157	47835	47535	44156	43815
359160F354795	S19	Botanical Garden	44702	43457	41863	41829	37694	37347
359161F354796	S21	Botanical Garden	41429	40080	38834	38457	35480	35177
359162F354797	T32	Parco Natura Viva	36328	35358	34202	34025	31532	31378
359163F354798	T33	Parco Natura Viva	37413	36499	35383	35240	32980	32723
359164F354799	T52	Parco Natura Viva	47644	46401	45224	44910	42018	41749
359165F354800	T53	Parco Natura Viva	34330	33396	32446	32332	30364	30205
359166F354801	BLB	Parco Natura Viva	33263	32038	30825	30790	27973	27870
359167F354802	PRS	Parco Natura Viva	30501	29647	28338	28402	25579	25405

Table S2. Mean relative abundances and standard deviation for every taxonomic rank.

Phylum	meanRA	sdRA
Bacteroidetes	38.21%	1.80%
Firmicutes	34.08%	0.29%
Spirochaetes	7.60%	0.90%
Proteobacteria	6.41%	1.06%
Tenericutes	2.72%	0.22%
Actinobacteria	2.56%	0.35%
Euryarchaeota	1.82%	0.30%
Verrucomicrobia	1.66%	0.43%
NA	1.29%	0.48%
Fibrobacteres	1.26%	1.30%
Synergistetes	0.54%	0.13%
Cyanobacteria	0.50%	0.08%
Chloroflexi	0.25%	0.13%
Lentisphaerae	0.21%	0.04%
Planctomycetes	0.16%	0.07%
Elusimicrobia	0.14%	0.15%
Patescibacteria	0.12%	0.07%
Kiritimatiellaeota	0.11%	0.04%
Fusobacteria	0.09%	0.33%
Epsilonbacteraeota	0.09%	0.27%
Acidobacteria	0.08%	0.04%
WPS-2	0.05%	0.11%
Deinococcus-Thermus	0.02%	0.13%
Nitrospirae	0.02%	0.12%
Gemmatimonadetes	0.01%	0.02%
Deferribacteres	0.00%	0.01%

Class	meanRA	sdRA
Bacteroidia	38.21%	1.80%
Clostridia	29.59%	0.20%
Spirochaetia	7.35%	0.92%
Gammaproteobacteria	4.23%	1.65%
Bacilli	3.32%	1.21%
Mollicutes	2.72%	0.22%
Actinobacteria	2.10%	0.42%
Verrucomicrobiae	1.66%	0.43%
NA	1.56%	0.41%
Alphaproteobacteria	1.40%	0.16%
Fibrobacteria	1.26%	1.30%
Thermoplasmata	1.01%	0.36%
Erysipelotrichia	0.78%	0.16%
Methanomicrobia	0.72%	0.25%
Deltaproteobacteria	0.68%	0.17%

Synergistia	0.54%	0.13%
Melainabacteria	0.43%	0.08%
Negativicutes	0.30%	0.16%
Acidimicrobiia	0.26%	0.12%
MVP-15	0.25%	0.21%
Lentisphaeria	0.17%	0.03%
Planctomycetacia	0.16%	0.07%
Coriobacteriia	0.13%	0.08%
Chloroflexia	0.12%	0.12%
Endomicrobia	0.12%	0.17%
Kiritimatiellae	0.11%	0.04%
Fusobacteriia	0.09%	0.33%
Campylobacteria	0.09%	0.27%
Anaerolineae	0.09%	0.16%
Methanobacteria	0.09%	0.08%
Saccharimonadia	0.08%	0.07%
Oxyphotobacteria	0.06%	0.08%
Thermoleophilia	0.06%	0.07%
Subgroup_6	0.04%	0.04%
Gracilibacteria	0.03%	0.07%
BRH-c20a	0.02%	0.02%
Gitt-GS-136	0.02%	0.07%
Deinococci	0.02%	0.13%
Elusimicrobia	0.02%	0.07%
Nitrospira	0.02%	0.12%
Acidobacteriia	0.01%	0.01%
Subgroup_17	0.01%	NA
KD4-96	0.01%	0.01%
Blastocatellia_(Subgroup_4)	0.01%	0.07%
Rs-M47	0.01%	0.01%
Gemmatimonadetes	0.01%	0.00%
Longimicrobia	0.01%	NA
Microgenomatia	0.01%	0.01%
Rhodothermia	0.004%	NA
JG30-KF-CM66	0.004%	NA
Deferribacteres	0.002%	0.013%
BD2-11_terrestrial_group	0.002%	NA
Parcubacteria	0.001%	NA

Order	**meanRA**	**sdRA**
Bacteroidales	36.55%	1.88%
Clostridiales	29.53%	0.20%

Spirochaetales	7.35%	0.92%
Bacillales	3.02%	1.32%
NA	2.99%	0.39%
Betaproteobacteriales	1.69%	2.41%
Pseudomonadales	1.31%	2.32%
Fibrobacterales	1.26%	1.30%
Izimaplasmatales	1.15%	0.23%
Methanomassiliicoccales	1.01%	0.36%
Micrococcales	0.90%	0.36%
Rhodospirillales	0.89%	0.21%
Pedosphaerales	0.79%	0.46%
Erysipelotrichales	0.78%	0.16%
Methanomicrobiales	0.72%	0.25%
Flavobacteriales	0.57%	0.53%
T2WK15B57	0.57%	0.24%
Propionibacteriales	0.55%	0.53%
Synergistales	0.54%	0.13%
Gastranaerophilales	0.43%	0.08%
Desulfovibrionales	0.40%	0.16%
Xanthomonadales	0.39%	0.38%
Mollicutes_RF39	0.39%	0.05%
Enterobacteriales	0.34%	0.23%
Kineosporiales	0.32%	0.70%
Verrucomicrobiales	0.32%	0.84%
Lactobacillales	0.31%	0.32%
Selenomonadales	0.30%	0.16%
Opitutales	0.27%	0.09%
Aeromonadales	0.25%	0.20%
Anaeroplasmatales	0.23%	0.11%
Chitinophagales	0.20%	0.14%
Mycoplasmatales	0.20%	1.12%
Victivallales	0.17%	0.03%
Microtrichales	0.17%	0.12%
Pirellulales	0.16%	0.07%
Actinomycetales	0.15%	0.46%
Sphingobacteriales	0.15%	0.15%
Coriobacteriales	0.13%	0.08%
Pasteurellales	0.13%	0.25%
Rhizobiales	0.13%	0.06%
Rickettsiales	0.12%	0.11%

Bradymonadales	0.12%	0.29%
Endomicrobiales	0.12%	0.17%
Thermomicrobiales	0.12%	0.12%
WCHB1-41	0.11%	0.04%
Corynebacteriales	0.11%	0.06%
LD1-PB3	0.11%	0.30%
Rhodobacterales	0.10%	0.10%
Fusobacteriales	0.09%	0.33%
Campylobacterales	0.09%	0.27%
Methanobacteriales	0.09%	0.08%
Anaerolineales	0.08%	0.17%
Actinomarinales	0.08%	0.13%
DMI	0.08%	0.08%
Saccharimonadales	0.08%	0.07%
Sphingomonadales	0.08%	0.13%
Desulfuromonadales	0.07%	0.20%
Chloroplast	0.06%	0.08%
Myxococcales	0.06%	0.03%
Solirubrobacterales	0.05%	0.07%
Bacteroidetes_VC2.1_Bac22	0.03%	0.03%
Alteromonadales	0.03%	0.04%
Absconditabacteriales_(SR1)	0.03%	0.08%
Oceanospirillales	0.03%	NA
Cytophagales	0.03%	0.07%
Frankiales	0.02%	0.12%
Deinococcales	0.02%	0.13%
Elusimicrobiales	0.02%	0.07%
Nitrospirales	0.02%	0.12%
Tistrellales	0.02%	0.03%
Gaiellales	0.02%	0.06%
Bdellovibrionales	0.01%	0.09%
Caulobacterales	0.01%	0.02%
Bifidobacteriales	0.01%	0.06%
CCD24	0.01%	0.09%
PLTA13	0.01%	0.07%
Pseudonocardiales	0.01%	0.03%
Steroidobacterales	0.01%	0.01%
Blastocatellales	0.01%	0.07%
Acetobacterales	0.01%	0.01%
Streptomycetales	0.01%	NA

Chloroflexales	0.01%	0.05%
Micromonosporales	0.01%	0.03%
Solibacterales	0.01%	0.00%
Syntrophobacterales	0.01%	NA
Cellvibrionales	0.01%	NA
Elsterales	0.01%	0.02%
R7C24	0.01%	NA
Gemmatimonadales	0.01%	0.00%
Orbales	0.01%	NA
Longimicrobiales	0.01%	NA
Candidatus_Pacebacteria	0.01%	0.01%
Caldilineales	0.004%	NA
Paracaedibacterales	0.004%	0.001%
Rhodothermales	0.004%	NA
Gammaproteobacteria_Incertae_Sedis	0.004%	NA
Chthoniobacterales	0.004%	0.006%
Subgroup_2	0.004%	NA
Streptosporangiales	0.003%	NA
EMP-G18	0.003%	0.008%
Acidobacteriales	0.003%	NA
NB1-j	0.003%	NA
211ds20	0.003%	NA
Deferribacterales	0.002%	0.013%
Ardenticatenales	0.002%	NA
Desulfobacterales	0.002%	0.007%
Candidatus_Nomurabacteria	0.001%	NA
RCP2-54	0.001%	NA
Coxiellales	0.001%	NA

Family	**meanRA**	**sdRA**
NA	17.46%	0.78%
Ruminococcaceae	14.09%	0.22%
Rikenellaceae	12.09%	1.20%
Spirochaetaceae	7.35%	0.92%
vadinHA21	6.71%	7.95%
Lachnospiraceae	6.07%	0.22%
Clostridiales_vadinBB60_group	3.58%	0.18%
M2PB4-65_termite_group	2.70%	1.45%
Paludibacteraceae	2.61%	1.94%
Christensenellaceae	2.29%	0.13%
Bacillaceae	2.05%	1.44%
Prevotellaceae	1.42%	1.42%

Neisseriaceae	1.24%	8.23%
Clostridiaceae_1	1.24%	0.29%
Moraxellaceae	1.18%	3.10%
Methanomethylophilaceae	1.01%	0.36%
possible_family_01	0.81%	2.30%
Pedosphaeraceae	0.79%	0.46%
Erysipelotrichaceae	0.78%	0.16%
Methanocorpusculaceae	0.72%	0.25%
Family_XIII	0.58%	0.17%
Synergistaceae	0.54%	0.13%
Weeksellaceae	0.50%	0.61%
Staphylococcaceae	0.49%	1.62%
Planococcaceae	0.48%	0.82%
Tannerellaceae	0.47%	0.34%
Nocardioidaceae	0.44%	0.62%
Burkholderiaceae	0.43%	0.24%
Fibrobacteraceae	0.43%	0.26%
Desulfovibrionaceae	0.40%	0.16%
Xanthomonadaceae	0.38%	0.40%
Bacteroidaceae	0.36%	0.17%
Marinilabiliaceae	0.34%	0.44%
Enterobacteriaceae	0.34%	0.23%
COB_P4-1_termite_group	0.32%	0.57%
Kineosporiaceae	0.32%	0.70%
Intrasporangiaceae	0.29%	0.14%
Peptostreptococcaceae	0.28%	0.14%
Puniceicoccaceae	0.26%	0.09%
Lactobacillaceae	0.26%	0.38%
Acidaminococcaceae	0.25%	0.19%
Succinivibrionaceae	0.24%	0.21%
Akkermansiaceae	0.24%	1.32%
Anaeroplasmataceae	0.23%	0.11%
Family_XI	0.22%	0.41%
Mycoplasmataceae	0.20%	1.12%
Dermatophilaceae	0.19%	0.37%
Chitinophagaceae	0.18%	0.15%
Bacteroidales_UCG-001	0.17%	0.25%
Brevibacteriaceae	0.16%	1.25%
Pirellulaceae	0.16%	0.07%
Actinomycetaceae	0.15%	0.46%
F082	0.14%	0.07%
Peptococcaceae	0.14%	0.04%
Pasteurellaceae	0.13%	0.25%
Pseudomonadaceae	0.13%	0.13%
Endomicrobiaceae	0.12%	0.17%
Ilumatobacteraceae	0.12%	0.15%
JG30-KF-CM45	0.12%	0.12%

Propionibacteriaceae	0.11%	0.18%
GZKB124	0.11%	0.08%
Rhodobacteraceae	0.10%	0.10%
Heliobacteriaceae	0.10%	0.19%
Campylobacteraceae	0.09%	0.27%
Porphyromonadaceae	0.09%	0.24%
Methanobacteriaceae	0.09%	0.08%
Anaerolineaceae	0.08%	0.17%
Leptotrichiaceae	0.08%	0.26%
Sphingomonadaceae	0.08%	0.13%
Microbacteriaceae	0.08%	0.24%
Rickettsiaceae	0.06%	0.15%
Flavobacteriaceae	0.06%	0.13%
Veillonellaceae	0.05%	0.03%
Rhizobiaceae	0.04%	0.03%
Eggerthellaceae	0.04%	0.13%
Victivallaceae	0.04%	0.05%
Coriobacteriales_Incertae_Sedis	0.04%	0.03%
p-251-o5	0.04%	0.04%
Dermabacteraceae	0.04%	0.11%
Dysgonomonadaceae	0.04%	0.11%
Iamiaceae	0.04%	0.05%
Eubacteriaceae	0.04%	0.03%
vadinBE97	0.04%	0.02%
Saccharimonadaceae	0.04%	0.03%
Corynebacteriaceae	0.04%	0.05%
Streptococcaceae	0.04%	0.16%
Promicromonosporaceae	0.04%	0.07%
Beutenbergiaceae	0.03%	0.15%
Dietziaceae	0.03%	0.03%
Hyphomicrobiaceae	0.03%	0.11%
Terasakiellaceae	0.03%	0.04%
Micrococcaceae	0.03%	0.04%
67-14	0.03%	0.05%
Halomonadaceae	0.03%	NA
Sandaracinaceae	0.02%	0.04%
PeH15	0.02%	NA
Mycobacteriaceae	0.02%	0.07%
Solirubrobacteraceae	0.02%	0.10%
Bogoriellaceae	0.02%	0.07%
Saprospiraceae	0.02%	0.06%
Nocardiaceae	0.02%	0.08%
DEV007	0.02%	0.11%
Shewanellaceae	0.02%	0.01%
Deinococcaceae	0.02%	0.13%
Elusimicrobiaceae	0.02%	0.07%
Xanthobacteraceae	0.02%	0.10%

Geodermatophilaceae	0.02%	NA
Nitrospiraceae	0.02%	0.12%
Fusobacteriaceae	0.02%	0.06%
Sphingobacteriaceae	0.02%	0.05%
Geminicoccaceae	0.02%	0.03%
D05-2	0.01%	0.02%
Muribaculaceae	0.01%	0.02%
Microscillaceae	0.01%	0.02%
Bifidobacteriaceae	0.01%	0.06%
Pseudonocardiaceae	0.01%	0.03%
Spirosomaceae	0.01%	NA
Alteromonadaceae	0.01%	0.01%
Bacteriovoracaceae	0.01%	NA
Myxococcaceae	0.01%	0.03%
Blastocatellaceae	0.01%	0.07%
Cellulomonadaceae	0.01%	NA
Beijerinckiaceae	0.01%	0.01%
Acetobacteraceae	0.01%	0.01%
Streptomycetaceae	0.01%	NA
Chloroflexaceae	0.01%	0.05%
Amb-16S-1323	0.01%	NA
Micromonosporaceae	0.01%	0.03%
Hyphomonadaceae	0.01%	0.01%
Solibacteraceae_(Subgroup_3)	0.01%	0.00%
Archangiaceae	0.01%	0.01%
Syntrophaceae	0.01%	NA
Microbulbiferaceae	0.01%	NA
Enterococcaceae	0.01%	NA
BIrii41	0.01%	0.03%
Rhodanobacteraceae	0.01%	0.02%
Gemmatimonadaceae	0.01%	0.00%
Orbaceae	0.01%	NA
Aeromonadaceae	0.01%	NA
Nakamurellaceae	0.01%	NA
Microtrichaceae	0.01%	0.00%
Woeseiaceae	0.01%	0.00%
Steroidobacteraceae	0.01%	0.01%
Nannocystaceae	0.01%	0.01%
Syntrophomonadaceae	0.01%	0.01%
Crocinitomicaceae	0.01%	0.01%
Caulobacteraceae	0.01%	NA
Longimicrobiaceae	0.01%	NA
TRA3-20	0.004%	NA
Aerococcaceae	0.004%	0.001%
Caldilineaceae	0.004%	NA
Paracaedibacteraceae	0.004%	NA
Rhodothermaceae	0.004%	0.006%

Unknown_Family	0.004%	NA
Xiphinematobacteraceae	0.003%	NA
Bdellovibrionaceae	0.003%	0.008%
Thermomonosporaceae	0.003%	NA
Rhodocyclaceae	0.003%	NA
Devosiaceae	0.003%	NA
Acidobacteriaceae_(Subgroup_1)	0.002%	0.013%
Rubritaleaceae	0.002%	NA
Dermacoccaceae	0.002%	0.007%
Methyloligellaceae	0.001%	NA
Deferribacteraceae	0.001%	NA
Ardenticatenaceae	0.001%	NA
Desulfobulbaceae	0.004%	NA
Leuconostocaceae	0.004%	0.001%
Coxiellaceae	0.004%	NA

Genus	**meanRA**	**sdRA**
NA	47.67%	0.92%
Treponema_2	6.27%	1.05%
Rikenellaceae_RC9_gut_group	4.94%	1.10%
DMER64	3.44%	1.90%
Ruminococcaceae_UCG-010	3.26%	0.11%
Paludibacter	2.12%	3.74%
Bacillus	2.05%	1.44%
Christensenellaceae_R-7_group	1.81%	0.12%
Ruminococcaceae_UCG-014	1.62%	0.16%
Acetobacteroides	1.43%	1.54%
Ruminococcus_1	1.34%	0.50%
Ruminococcaceae_NK4A214_group	1.24%	0.15%
Acinetobacter	1.18%	3.10%
Alloprevotella	1.01%	3.50%
dgA-11_gut_group	0.86%	1.49%
Methanocorpusculum	0.72%	0.25%
Anaerosporobacter	0.70%	0.25%
Lachnospiraceae_UCG-007	0.70%	0.26%
Blvii28_wastewater-sludge_group	0.51%	0.59%
Staphylococcus	0.49%	1.62%
Clostridium_sensu_stricto_1	0.49%	0.24%
Alistipes	0.48%	0.16%
Sarcina	0.48%	0.38%
Chryseobacterium	0.43%	0.77%
Nocardioides	0.43%	0.63%
Fibrobacter	0.43%	0.26%
Solibacillus	0.42%	1.18%
Cellulosilyticum	0.39%	0.15%
Lachnoclostridium_10	0.39%	0.50%
Macellibacteroides	0.37%	0.42%

Bacteroides	0.36%	0.17%
Acetivibrio	0.34%	0.89%
Desulfovibrio	0.31%	0.20%
Lactobacillus	0.26%	0.38%
Clostridium_sensu_stricto_3	0.24%	0.26%
Akkermansia	0.24%	1.32%
Erysipelotrichaceae_UCG-004	0.24%	0.10%
Fastidiosipila	0.24%	0.85%
Anaeroplasma	0.23%	0.11%
Papillibacter	0.21%	0.06%
Oscillibacter	0.21%	0.09%
Quadrisphaera	0.21%	0.96%
Breznakia	0.21%	0.36%
Family_XIII_UCG-001	0.20%	0.45%
Mycoplasma	0.20%	1.12%
Faecalibacterium	0.19%	0.14%
Terrisporobacter	0.19%	0.15%
Candidatus_Soleaferrea	0.18%	0.10%
Intestinimonas	0.18%	0.09%
Prevotellaceae_Ga6A1_group	0.18%	0.15%
Cerasicoccus	0.18%	0.11%
Brevibacterium	0.16%	1.25%
Escherichia/Shigella	0.15%	0.25%
Caproiciproducens	0.15%	0.31%
Ruminococcaceae_UCG-013	0.15%	0.06%
Ruminococcaceae_UCG-005	0.15%	0.09%
Anaerovorax	0.15%	0.04%
Prevotellaceae_UCG-004	0.14%	0.17%
Phascolarctobacterium	0.14%	0.28%
Chelonobacter	0.13%	0.25%
Pseudomonas	0.13%	0.13%
Gallicola	0.13%	1.16%
Arcanobacterium	0.13%	0.57%
Sphaerochaeta	0.13%	0.09%
Lysobacter	0.12%	0.22%
Ornithinimicrobium	0.12%	0.18%
Sediminispirochaeta	0.12%	0.08%
Ruminobacter	0.12%	0.29%
Candidatus_Endomicrobium	0.12%	0.17%
Pyramidobacter	0.11%	0.11%
Sutterella	0.11%	0.04%
Ruminiclostridium_6	0.10%	0.09%
Succinivibrio	0.10%	0.15%
Luteimonas	0.10%	0.17%
CPla-4_termite_group	0.10%	0.10%
Citrobacter	0.10%	0.25%
Lachnospira	0.10%	0.13%

Hydrogenispora	0.10%	0.19%
Campylobacter	0.09%	0.27%
Acidaminococcus	0.09%	0.08%
Lachnospiraceae_NK4A136_group	0.09%	0.10%
Ruminococcaceae_UCG-008	0.09%	0.20%
Ottowia	0.08%	0.22%
Bilophila	0.08%	0.06%
Anaerocolumna	0.08%	0.21%
Erysipelatoclostridium	0.08%	0.06%
Flexilinea	0.08%	0.18%
Paracoccus	0.07%	0.11%
Propioniciclava	0.07%	0.17%
Intestinibacter	0.07%	0.14%
Parabacteroides	0.06%	0.07%
Butyrivibrio	0.06%	0.28%
Klebsiella	0.06%	0.18%
Sedimentibacter	0.06%	0.05%
Anaerocella	0.06%	0.19%
Methanobrevibacter	0.06%	0.10%
Hydrogenoanaerobacterium	0.05%	0.04%
Ruminococcus_2	0.05%	0.11%
Porphyromonas	0.05%	0.31%
Kribbia	0.05%	0.12%
Ruminococcaceae_UCG-002	0.05%	0.02%
Lachnospiraceae_UCG-009	0.05%	0.11%
Flavonifractor	0.05%	0.05%
Pseudoclavibacter	0.05%	0.41%
Janibacter	0.04%	0.16%
Roseburia	0.04%	0.09%
Anaerostipes	0.04%	0.07%
Ornithobacterium	0.04%	0.10%
Brachybacterium	0.04%	0.11%
Filobacterium	0.04%	0.13%
Iamia	0.04%	0.05%
Tannerella	0.04%	0.06%
Limnobacter	0.04%	0.13%
Anaerofustis	0.04%	0.03%
Enterorhabdus	0.04%	0.14%
Serinibacter	0.03%	0.15%
p-1088-a5_gut_group	0.03%	0.02%
Dietzia	0.03%	0.03%
Ilumatobacter	0.03%	0.08%
Thermomonas	0.03%	0.11%
Taibaiella	0.03%	0.12%
Aestuariispira	0.03%	0.04%
Haoranjiania	0.03%	0.08%
Erysipelothrix	0.03%	0.04%

Ruminococcaceae_V9D2013_group	0.03%	0.14%
F0058	0.03%	NA
Kushneria	0.03%	NA
Proteiniphilum	0.03%	0.09%
Corynebacterium	0.03%	0.06%
Tessaracoccus	0.03%	0.13%
Streptococcus	0.03%	0.25%
Pirellula	0.03%	0.04%
Caryophanon	0.03%	0.04%
Family_XIII_AD3011_group	0.02%	0.02%
GWE2-31-10	0.02%	0.06%
Isoptericola	0.02%	0.10%
W5053	0.02%	0.10%
Lachnospiraceae_FCS020_group	0.02%	0.09%
Lysinibacillus	0.02%	0.03%
Oxalobacter	0.02%	0.06%
Mobiluncus	0.02%	0.12%
Pedomicrobium	0.02%	0.14%
Mycobacterium	0.02%	0.07%
Conexibacter	0.02%	0.10%
Georgenia	0.02%	0.07%
Ruminiclostridium_1	0.02%	0.02%
Tetrasphaera	0.02%	NA
Altererythrobacter	0.02%	0.04%
Anaerobiospirillum	0.02%	0.03%
Herbinix	0.02%	0.18%
Lachnospiraceae_UCG-010	0.02%	0.02%
Agromyces	0.02%	0.10%
Subdoligranulum	0.02%	0.03%
hoa5-07d05_gut_group	0.02%	0.05%
Flavobacterium	0.02%	0.13%
Mesorhizobium	0.02%	0.03%
Shewanella	0.02%	0.01%
Deinococcus	0.02%	0.13%
Elusimicrobium	0.02%	0.07%
OLB8	0.02%	0.06%
Butyricicoccus	0.02%	0.09%
Antricoccus	0.02%	NA
Nitrospira	0.02%	0.12%
Niabella	0.01%	0.14%
Cloacibacillus	0.01%	NA
Fonticella	0.01%	0.07%
Fusobacterium	0.01%	0.08%
H1	0.01%	0.02%
Bifidobacterium	0.01%	0.06%
Ruminococcaceae_UCG-012	0.01%	0.03%
Pseudonocardia	0.01%	0.03%

Citricoccus	0.01%	NA
Rheinheimera	0.01%	0.01%
Terrimonas	0.01%	0.02%
Bergeyella	0.01%	0.03%
Clostridium_sensu_stricto_13	0.01%	0.03%
Comamonas	0.01%	NA
Leifsonia	0.01%	NA
Peredibacter	0.01%	NA
Marinilutecoccus	0.01%	0.11%
U29-B03	0.01%	NA
Robiginitalea	0.01%	NA
UBA1819	0.01%	0.03%
Proteus	0.01%	NA
Cellulosimicrobium	0.01%	NA
GCA-900066755	0.01%	NA
Romboutsia	0.01%	0.04%
Corynebacterium_1	0.01%	NA
Candidatus_Methanogranum	0.01%	0.02%
Pseudactinotalea	0.01%	NA
Mucilaginibacter	0.01%	0.08%
Lactococcus	0.01%	0.04%
Streptomyces	0.01%	NA
Intrasporangium	0.01%	NA
Anaerovibrio	0.01%	0.03%
Candidatus_Chloroploca	0.01%	0.05%
Hyphomicrobium	0.01%	0.04%
Arthrobacter	0.01%	NA
Hespellia	0.01%	0.02%
Methanosphaera	0.01%	0.06%
Ruminococcaceae_UCG-007	0.01%	0.01%
Candidatus_Solibacter	0.01%	0.00%
Melittangium	0.01%	0.01%
XBB1006	0.01%	0.02%
Stenotrophobacter	0.01%	NA
Microbulbifer	0.01%	NA
Enterococcus	0.01%	NA
Petrimonas	0.01%	NA
Ornithinicoccus	0.01%	NA
Lachnospiraceae_NK4B4_group	0.01%	0.01%
Peptoanaerobacter	0.01%	0.03%
Chryseolinea	0.01%	0.00%
Angustibacter	0.01%	NA
Ruminococcaceae_UCG-009	0.01%	0.01%
Incertae_Sedis	0.01%	NA
Pseudofulvimonas	0.01%	0.02%
Lautropia	0.01%	NA
SWB02	0.01%	0.01%

Gilliamella	0.01%	NA
Aeromonas	0.01%	NA
Erythrobacter	0.01%	NA
Nakamurella	0.01%	NA
Capnocytophaga	0.01%	NA
Kribbella	0.01%	NA
Hydrogenophaga	0.01%	0.01%
Woeseia	0.01%	0.00%
Aggregicoccus	0.01%	0.03%
Pseudoxanthomonas	0.01%	NA
Verrucosispora	0.01%	NA
Rhodococcus	0.01%	NA
Roseiarcus	0.01%	NA
Gordonibacter	0.01%	0.02%
Fluviicola	0.01%	0.01%
Anaerobium	0.01%	NA
Qipengyuania	0.01%	NA
CL500-29_marine_group	0.01%	NA
Brevundimonas	0.01%	NA
GCA-900066225	0.01%	0.03%
Candidatus_Alysiosphaera	0.005%	NA
Tyzzerella	0.005%	0.017%
Sandaracinus	0.005%	NA
Amaricoccus	0.005%	NA
Aliihoeflea	0.005%	NA
Morganella	0.005%	NA
Lachnoclostridium_12	0.005%	NA
Rhodobacter	0.005%	NA
Myxococcus	0.005%	NA
Actinomyces	0.005%	NA
Ruminiclostridium_9	0.004%	NA
Enterobacter	0.004%	NA
Roseomonas	0.004%	NA
Microvirga	0.004%	NA
Sphingomonas	0.004%	0.009%
Novosphingobium	0.004%	NA
Acidibacter	0.004%	NA
Candidatus_Xiphinematobacter	0.004%	0.006%
Angelakisella	0.004%	0.011%
Candidatus_Methanoplasma	0.004%	NA
Vitellibacter	0.004%	0.007%
Bdellovibrio	0.004%	0.002%
Syntrophobotulus	0.003%	0.009%
Shinella	0.003%	NA
Sphingobacterium	0.003%	NA
Actinocorallia	0.003%	NA
Dielma	0.003%	NA

Psychrobacillus	0.003%	NA
Syntrophomonas	0.003%	NA
Geminicoccus	0.003%	NA
Nannocystis	0.003%	NA
Micromonospora	0.003%	NA
Clostridium_sensu_stricto_2	0.003%	NA
Prevotellaceae_UCG-001	0.003%	0.010%
Anaerolinea	0.003%	NA
Thauera	0.003%	NA
Devosia	0.003%	NA
IMCC26207	0.003%	NA
Occallatibacter	0.003%	NA
Gordonia	0.003%	NA
Roseibacillus	0.003%	NA
Oscillospira	0.003%	NA
Victivallis	0.003%	NA
Steroidobacter	0.003%	NA
Kytococcus	0.003%	NA
Murdochiella	0.003%	NA
Shuttleworthia	0.003%	NA
Brachymonas	0.002%	NA
Pseudorhodoplanes	0.002%	NA
Cellulosibacter	0.002%	NA
Mucispirillum	0.002%	0.013%
Parapedobacter	0.002%	NA
Pelospora	0.002%	NA
Bradyrhizobium	0.002%	NA
Anaerofilum	0.002%	NA
Blastocatella	0.002%	NA
Hirschia	0.002%	NA
Oceaniovalibus	0.002%	NA
Lachnospiraceae_AC2044_group	0.002%	NA
Desulfobulbus	0.002%	0.007%
Anaerosinus	0.002%	NA
Tyzzerella_3	0.002%	0.009%
Weissella	0.002%	NA
Peptoniphilus	0.002%	NA
Fournierella	0.002%	NA
Coprococcus_3	0.002%	NA
Mobilitalea	0.002%	NA
Catabacter	0.002%	NA
Lachnospiraceae_UCG-006	0.002%	NA
Sporobacter	0.002%	NA
Coxiella	0.001%	NA
Ruminiclostridium	0.001%	NA

Species	meanRA	sdRA
NA	99.384%	0.80%
Lactobacillus kunkeei	0.215%	0.46%
Clostridium butyricum	0.052%	0.08%
Acinetobacter lwoffii	0.033%	0.08%
Methylocella silvestris	0.028%	0.01%
Arthrospira maxima	0.028%	0.15%
Zymomonas mobilis	0.028%	0.07%
Halomonas avicenniae	0.027%	NA
Acinetobacter bohemicus	0.021%	NA
Campylobacter iguaniorum	0.021%	0.06%
Methylarcula marina	0.014%	NA
Nocardies asteroides	0.013%	0.06%
Pseudonocardia ammonioxydans	0.013%	0.03%
Comamonas jiangduensis	0.012%	NA
Anaeroplasma varium	0.010%	NA
Sphingomonas aestuarii	0.009%	NA
Flavihumibacter solisilvae	0.009%	NA
Shewanella putrefaciens	0.008%	NA
Luteimonas arsenica	0.008%	NA
Gilliamella apicola	0.006%	NA
Pseudoxanthomonas kaohsiungensis	0.006%	NA
Mesorhizobium thiogangeticum	0.006%	NA
Lactobacillus melliventris	0.005%	NA
Campylobacter fetus	0.005%	0.01%
Enterocossus faecalis	0.005%	NA
Morganella morganii	0.005%	NA
Gluconacetobacter Gluconicum	0.005%	NA
Clostridium beijerinckii	0.004%	NA
Sphingobium ummariense	0.004%	NA
Psychrobacillus psychrodurans	0.003%	NA
Micromonas pusilla	0.003%	NA
Micromonospora pattaloongensis	0.003%	NA
Thauera aminoaromatica	0.003%	NA
Prevotella intermedia	0.003%	NA
Mycobacterium conspicuum	0.002%	NA

Table S3. Results of LEfSe on differentially abundance taxa among location showing the *p* values, false discovery rate, mean abundance for each location. Data were normalized using Total Sum Scaling and LDA score (effect size).
[1]False Discovery Rate correction for multiple comparison, [2]Linear Discriminant Analysis score: estimate the effect size of each difference

Taxa		*p* values	[1]FDR values	Botanical Garden	Curieuse	Parco Natura viva	[2]LDA score
Family	vadinHA21	0.002	0.096	310150	103200	1731400	5.91
	Marinilabiliaceae	0.002	0.096	12479	890.7	93599	4.67
	Clostridiales_Family_XIII	0.004	0.096	137900	60284	20974	4.77
	Pedosphaeraceae	0.006	0.096	33728	37316	171460	4.84
	Peptostreptococcaceae	0.006	0.096	4448.1	63181	3757.6	4.47
	Clostridiaceae_1	0.006	0.096	45480	233150	45295	4.97
	Dysgonomonadaceae	0.007	0.096	2447.5	0	0	3.09
Genus	*Anaerocella*	0.000	0.045	35298	0	0	4.25
	Parabacteroides	0.001	0.045	0	0	10785	3.73
Species	*C. iguaniorum*	0.005	0.051	583.74	0	5818.8	3.46
ASV	ASV_1300	0.000	0.037	3603	0	0	3.26
	ASV_1239	0.000	0.037	3943.9	0	0	3.3
	ASV_1240	0.000	0.037	4142	0	0	3.32
	ASV_1150	0.000	0.037	5039.2	0	0	3.4
	ASV_1133	0.000	0.037	5115.7	0	0	3.41
	ASV_1038	0.000	0.037	6185	0	0	3.49
	ASV_1045	0.000	0.037	6219.9	0	0	3.49
	ASV_1019	0.000	0.037	6719	0	0	3.53
	ASV_955	0.000	0.037	6854.5	0	0	3.54
	ASV_872	0.000	0.037	8491.6	0	0	3.63
	ASV_862	0.000	0.037	9103.8	0	0	3.66
	ASV_822	0.000	0.037	9913.1	0	0	3.7
	ASV_498	0.000	0.037	17760	0	0	3.95
	ASV_295	0.000	0.037	35298	0	0	4.25
	ASV_191	0.000	0.037	50821	0	0	4.41
	ASV_632	0.001	0.037	0	0	7531.3	3.58
	ASV_471	0.001	0.037	0	0	10104	3.7
	ASV_425	0.001	0.037	0	0	10785	3.73
	ASV_391	0.001	0.037	0	0	11509	3.76
	ASV_134	0.001	0.037	0	0	34520	4.24
	ASV_101	0.001	0.037	0	0	50771	4.4
	ASV_41	0.001	0.037	0	0	78711	4.6
	ASV_40	0.001	0.037	0	0	100270	4.7
	ASV_108	0.001	0.041	7080.8	0	43817	4.34

Table S4. Differential expressed ASVs corresponding classification

ASVs	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV1300	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Hydrogenoanaerobacterium	NA
ASV1239	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_vadinBB60_group	NA	NA
ASV1240	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
ASV1150	Bacteria	Firmicutes	Clostridia	NA	NA	NA	NA
ASV1133	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_vadinBB60_group	NA	NA
ASV1038	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcaceae_NK4A214_group	NA
ASV1045	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
ASV1019	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
ASV955	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	NA
ASV872	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus_1	NA
ASV862	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
ASV822	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum	NA
ASV498	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponema_2	NA
ASV295	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcaceae_UCG-014	NA
ASV191	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
ASV632	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerosporobacter	mobilis
ASV471	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum	NA
ASV425	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila	NA
ASV391	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponema_2	NA
ASV134	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	NA	NA
ASV101	Archaea	Euryarchaeota	Thermoplasmata	Methanomassiliicoccales	Methanomethylophilaceae	NA	NA
ASV41	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut_group	NA
ASV40	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	NA
ASV108	Bacteria	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Xiphinematobacteraceae	Candidatus_Xiphinematobacter	NA

3. Gut Microbiota of Lemur: *Indri indri*

Conservation strategies require multidisciplinary approaches to monitor and protect primate populations, many of which are rapidly declining around the world. In particular, it is true for species that are endemic of a small area in the world such as Madagascar. A very recent update of the The IUCN Red List of Threatened Species™ from the International Union for Conservation of Nature (IUCN, 2020) shows that almost a third (31%) of all lemur species in Madagascar are now Critically Endangered, just one step away from extinction, with 98% of them threatened. This means that there are 33 lemur species listed as Critically Endangered, and 103 of the 107 surviving species threatened with extinction as a result of intensifying human pressures due to deforestation and illegal hunting. Among those there is *Indri indri* the largest living lemur of Madagascar. Indris are the most folivorous of all indriids and have morphological specialization for the consumption and digestion of leaves. No seeds are passed whole in the resulting fecal matter, which indicates that indri is not a seed disperser but a seed predator (Pollock 1977). The species shows a preference for immature leaves (72%) with a reduced emphasis on fruit seeds/whole fruits (16%) and flowers (7%) (Powzyk 1997). They also feed on bark, galls and mushrooms. Indris consume soil on a regular basis. The consumption of soil may be a means to combat the chemical deterrents often found in fruit seeds. Microbial ecology and next-generation microbiome analyses offer valuable perspectives and tools for investigating and monitoring primate health and improving conservation efforts. The microbial communities inhabiting primates and other taxa profoundly affect host health, nutrition, physiology, and immune systems. Microbial communities might be sensitive to alterations in the external environment and microbial diversity seems to correlate with habitat quality with direct health consequences. The application of microbial analyses to conservation is currently in its infancy but holds enormous potential.

To date, no conservation policy or legislation includes microbiome assessments. Integrating new understanding of the patterns of microbial diversity offer valuable tools for informing conservation strategies and monitoring and promoting primate health.

The following study provides an insight in the gut microbiota of *Indri indri* to improve the knowledge on the dependency of this species from the geophagy soil. I am working

in a zoological garden where a great attention has been given to this species. An exhibit has been built to explain that urgent actions should be done to preserve this species in Madagascar. In collaboration with University of Turin, the long-term Madagascar conservation project celebrated 20 years of conservation efforts in 2019. Since every effort to host the species in controlled environment has been failed, by involving experts in different fields and bring together different expertise, I have thought the following study to explore the importance of the link between the indris and their environment.

3.1 The second paper

Correa F., Torti V., Spiezio C., Checcucci A., Modesto M., Borruso L., Cavani L., Mimmo T., Cesco S., Luise D., Randrianarison R.M., Gamba M., Rarojoson N.J., Di Vito M., Bugli F.[8], Mattarelli P., Trevisi P., Giacoma C., **Sandri C.** 2020

Characterization of the faecal microbiome: a non-invasive tool for investigating the ecology of *Indri indri*, a threatened lemur species of Madagascar

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Disentangling the Possible Drivers of *Indri indri* Microbiome: A Threatened Lemur Species of Madagascar

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Research on the gut microbiome may help with increasing our understanding of primate health with species' ecology, evolution, and behavior. In particular, microbiome-related information has the potential to clarify ecology issues, providing knowledge in support of wild primates conservation and their associated habitats. Indri (*Indri indri*) is the largest extant living lemur of Madagascar. This species is classified as "critically endangered" by the IUCN Red List of Threatened Species, representing one of the world's 25 most endangered primates. Indris diet is mainly folivorous, but these primates frequently and voluntarily engage in geophagy. Indris have never been successfully bred under human care, suggesting that some behavioral and/or ecological factors are still not considered from the *ex situ* conservation protocols. Here, we explored gut microbiome composition of 18 indris belonging to 5 different family groups. The most represented phyla were Proteobacteria 40.1 ± 9.5%, Bacteroidetes 28.7 ± 2.8%, Synergistetes 16.7 ± 4.5%, and Firmicutes 11.1 ± 1.9%. Further, our results revealed that bacterial alpha and beta diversity were influenced by indri family group and sex. In addition, we investigated the chemical composition of geophagic soil to explore the possible ecological value of soil as a nutrient supply. The quite acidic pH and high levels of secondary oxide-hydroxides of the soils could play a role in the folivorous diet's gut detoxification activity. In addition, the high contents of iron and manganese found the soils could act as micronutrients in the indris' diet. Nevertheless, the concentration of a few elements (i.e., calcium, sulfur, boron, nickel, sodium, and chromium) was higher in non-geophagic than in geophagic soils. In conclusion, the data presented herein provide a baseline for outlining some possible drivers responsible for the gut microbiome diversity in indris, thus laying the foundations for developing further strategies involved in indris' conservation.

Keywords: gut microbiome, soil quality, non-human primate, animal ecology, endangered species, geophagy, forest ecology

INTRODUCTION

Studies on human and animal microbiome have provided compelling evidence that gut microbial diversity is fundamental in shaping metabolic and regulatory networks involved in the maintenance of host healthy status, as well as in a spectrum of disease states (Shreiner et al., 2015; Sandri et al., 2020). Indeed, the mammalian gut microbiome plays a crucial role in host physiology, supporting vitamin synthesis, helping in complex carbohydrates digestion, toxins metabolism, pathogens antagonism, and immune system modulation (Cresci and Bawden, 2015). Factors influencing the differences in mammalian gut microbiome are debated: host behaviors and environments, biogeography, and host genetic effects (e.g., gastrointestinal tract morphology) are of great importance (Lankau et al., 2012; Moeller et al., 2013; Amato et al., 2016). Previous studies showed that frequent social networks are positively associated with high similarity in gut microbial diversity (Tung et al., 2015; Perofsky et al., 2019). Vertical transmission from parent to offspring is the first driver for gut microbiome development, but horizontal transmission from the environment provides a crucial microbial colonization route. Even if microbial transmission due to sociality has traditionally been viewed as a risk for pathogen exposure, it may also be essential to host health. Therefore, it can avoid bottleneck-induced extinctions that could occur when the transmission of microorganisms is strict from parent to offspring. Indeed, it can allow the acquisition of beneficial microbes, particularly those that might not be gained through vertical transmission (Lombardo, 2008; Amaral et al., 2017). Moeller et al. (2013) underlined that gut microbial populations' social inheritance might be fundamental for preserving microbial diversity over evolutionary time scales.

The lemurs harbored species-specific and/or populations specific microbiomes, which are mainly influenced by their dietary specificity, even on a seasonal basis (Fogel, 2015; Greene et al., 2020). Globally, host habitat is one of the most important factors for gut microbiome modulation, and recently, increasing attention has been devoted to the soil. Indeed, a recent study (Grieneisen et al., 2019) on the gut microbiome of terrestrially living baboons showed that bare soil exceeds 15 times the predictive ability of host genetics in shaping the gut microbiome. Studies in mice (Li et al., 2016; Zhou et al., 2018) confirmed that the effect of soil on gut microbiome composition is comparable to that exerted by diet. Therefore, these studies suggest that contact/ingestion of soil components is beneficial for a healthy gut microbiome.

Indri indri is the largest extant living lemur (**Figure 1** and **Supplementary Video 1**). It is mainly arboreal and is the only lemur that communicates using songs. Indris songs mediate both intra- and inter-group communication (Torti et al., 2013) and relay information regarding individual features (i.e., sex and age) (De Gregorio et al., 2019, 2021). This species has never successfully been kept in a controlled environment and it is considered one of the Malagasy most critically endangered lemurs according to the IUCN Red List of Threatened Species (King et al., 2020), representing one of the world's 25 most endangered primates (Torti et al., 2019). This species is also listed in Appendix I of CITES (Heinen and Mehta, 1999). Indris are territorial, socially primates living in small family groups (Pollock, 1979; Bonadonna et al., 2019), generally consisting of an adult male and female with their related offspring (2–6 individuals) (Torti et al., 2013; Gamba et al., 2016).

Non-human primates are characterized by many dietary specializations (Campbell, 2017). In particular, the ability to consume leaves is typical of new world monkeys (e.g., howler monkeys), old world monkeys (e.g., colobines), apes (e.g., gorillas), and also prosimians (e.g., indris, bamboo lemurs, and sportive lemurs). Indri is the most specialized folivorous among lemurs and, as such, has the highest degree of morphological specialization for leaves' consumption and digestion. Leaves contain carbohydrates, including cellulose and hemicellulose, and secondary metabolites, including toxic ones such as tannins and phenolics (Norconk et al., 2009). Indris are characterized by the typical morphology and anatomical specializations of folivorous primates, such as hypertrophic salivary glands, voluminous stomachs, sacculated caeca, and looped colons that facilitate efficient fermentation of leaf matter (Greene et al., 2020). The species shows a preference for immature leaves (72%) with a reduced emphasis on fruit seeds/whole fruits (16%) and flowers (7%) (Powzyk, 1997). Leaves and fruit seeds could contain toxic compounds varying in percentage depending on the season, maturity, etc. (Pebsworth et al., 2019). In addition, indris perform geophagy by consuming soil intentionally (Britt et al., 2002; Borruso et al., 2021). Some evidence suggests that geophagy is an adaptive behavior to protect from ingested toxic compounds and mineral supplementation as it facilitates consumption of plants binding toxic plant secondary compounds (PSCs) (Pebsworth et al., 2019). As a result of metabolic activity, plants with relevant antioxidant properties produce primary and secondary compounds. Nevertheless, several metabolites are universally distributed in many plant species; some are unique to individual plant cultivars and fill essential functions (Geilfus, 2019).

Studies regarding geophagy across non-human primates revealed that they eat items high in PSCs. Furthermore, they consume soil more often than sympatric populations, suggesting a decrease in gastrointestinal distress caused by PSCs. Geophagy can help the utilization of dietary resources high in PSCs, expanding the range of dietary components (Overdorff, 1993; Bocian, 1997; Powzyk and Mowry, 2003; Dew, 2005; Pebsworth et al., 2019). In addition to dietary toxins, mineral deficiencies, diarrhea, and altered gut pH were reported to cause geophagy (Krishnamani and Mahaney, 2000; Ferrari et al., 2008; Young et al., 2011). As these processes are not necessarily mutually exclusive, geophagy can play different functions, such as rare element supplementation, detoxification, and protection (Davies and Baillie, 1988; Huffman et al., 1997; Krishnamani and Mahaney, 2000; Pebsworth et al., 2019). Interestingly, geophagic soil could also be a reservoir for microbial species affecting indris' gut microbiome (Borruso et al., 2021). The highly specialized diet, physiology, and morphology of indri's gut may contribute to their susceptibility in a human-controlled environment. This is in analogy for what has been described for other endangered folivorous primate whose breeding was unsuccessful (Hale et al., 2018, 2019).

FIGURE 1 | Distribution of the territories occupied by the indri family groups sampled (main figure, **d**) and composition of each single group. Both adult and youngster indris, both sexes, feed on leaves **(a,b)** and perform geophagic behavior, eating soil in specific sites **(c)**.

Understanding the drivers of the gut indris microbiome and their relationship to the soil could be essential for planning strategies to conserve, monitor, and promote their health. Whether the gut microbiome facilitates the use of these hard-to-digest food items, it would be crucial to characterize the bacterial gut microbiome's shaping factors. Therefore, our work aimed to analyze: (1) the gut microbiome composition of wild indris belonging to five different familiar groups in Maromizaha, eastern Madagascar; (2) the potential drivers affecting host-microbial diversity, including sex, family group, and age class (3) the chemical composition of geophagic and non-geophagic soil, to unravel the possible adaptive ecological value as nutrient supply.

MATERIALS AND METHODS

Fecal and Soil Samples Collection

Fecal and soil samples were collected in a very narrow temporal window (between December 4th and 6th, 2018) to avoid confounding potential seasonal effects. Individual fecal samples were obtained from 18 indris (fecal material) belonging to 5 different social family groups (**Table 1** and **Figure 1**) (latitude 18°57′S and 19°00′S, longitude 48°26′E and 48°31′E, Madagascar). The samples were collected immediately after defecation, when only one animal, recognized using natural marks (Torti et al., 2013), was present. This procedure was essential to avoid individual misidentification during the sampling process (Bonadonna et al., 2019). Approximately 5 g of fecal samples were collected from each of the 18 individuals (**Table 1**) following the procedure described in Borruso et al. (2021).

Each sample was classified according to the following categories: sex, family group, and age class (Adult > 6 years and Juvenile < 6 years) (**Table 1** and **Figure 1**). In addition, soil samples were collected from seven geophagic and seven non-geophagic (control) sites. All the geophagic sites were at the bases of trees uprooted by wind or rainfall, with the lower soil horizons exposed. We noted the location (waypoint) during soil-eating events, and we followed behaviors before and after the geophagy event. Control sites were selected from areas with the same characteristics (slope, vegetation, etc.) and located at less than 20 m from geophagic sites after removing the superficial soil layer to sample the same soil layer of the geophagic sites. The presence of the superficial layer together with debris proved that the groups have never used the control locations to consume soil. All samples were maintained in a portable cooler with ice packs before arrival at the lab.

Soil Characterization

Soil samples were air-dried, milled, and sieved at 2 mm for soil analysis in agreement with Soil Science Society of America (SSSA) methods (Sparks et al., 1996). Briefly, pH was determined in water (1:2.5, m/V), total carbon (C), and total nitrogen (N) using an elemental analyzer (CHNS-O Elemental Analyzer 1110, Thermo Scientific GmbH, Germany). Pseudo total element concentrations were determined after acid mineralization with aqua regia and hydrogen peroxide in an Ethos TC microwave lab station (Milestone, Italy) using an inductively coupled plasma

TABLE 1 | Description of each indri individual including sex, class age (Adult, >6 years; Juvenile, <6 years; NA*, not available) and family group, bacterial observed richness, and bacterial Shannon index values.

Samples ID	Sex	Class age	Family group	Observed richness	Shannon
L	Female	Adult	1MZ	44	2.87
M	Female	Juvenile	1MZ	41	2.55
O2	Male	Adult	1MZ	43	2.11
R	Female	Juvenile	2MZ	44	2.55
N2	NA*	Juvenile	2MZ	44	2.76
P	Male	Adult	2MZ	46	2.67
Q	Female	Adult	2MZ	47	2.90
G	Female	Juvenile	3MZ	35	2.55
H	Male	Juvenile	3MZ	37	1.95
I	Female	Adult	3MZ	35	2.41
C	Female	Adult	4MZ	38	2.70
E2	Male	Adult	4MZ	45	2.40
K	Female	Adult	6MZ	47	2.75
S	Male	Adult	6MZ	39	2.59
A2	Female	Adult	8MZ	55	2.94
B2	Male	Juvenile	8MZ	58	2.69
D2	Male	Adult	8MZ	55	2.62
F2	Male	Adult	8MZ	58	2.89

optical emission spectrometer (ICP-OES, Ametek Spectro, Arcos, Germany). Available metals were determined by ICP-OES after extraction for 2 h with 1 mol L^{-1} ammonium nitrate (NH_4NO_3) solution (1:2.5, m/V).

DNA Extraction and NGS Sequencing

Total DNA was isolated and extracted from indri fecal samples with DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) with slight modifications. Briefly, the lysis step was enhanced using a bead-beater (FastPrep 24G, MP Biomedicals, France), in which the "Powerbead" tubes containing the pellets (250 mg of fecal sample) and 800 μL of CD1 solution were subjected to two cycles of bead-beating at a speed of 4 m/s for 60 s with 45 s pause between cycles. The final elution volume was 100 μL in water. DNA was checked for purity (absorbance ratio 260/280 and 260/230) by spectrophotometry using NanoDrop (Fisher Scientific, 13 Schwerte, Germany) and quantified with the fluorometer Qubit® 2.0 (Invitrogen, Italy). Next, the DNA concentration of each sample was normalized to 1 ng μL^{-1}. The PCR was performed amplifying the V3–V4 region of the 16S rRNA gene (∼460 bp) with the primers Pro341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′) and Pro805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014), using Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy). The thermal cycling protocol consisted of the following conditions: initial denaturation at 94°C for 1′, followed by 25 cycles of denaturation at 94°C for 30″, annealing at 55°C for 30″, and extension 65°C for 45″, ending with 1 cycle at 68°C for 7′. Further, PCR samples were sent to BMR-Genomics Ltd., that according to the standard protocols carried out the other steps of the workflow and finally sequenced the libraries using a MiSq platform (300 × 2 bp) (Illumina Inc., San Diego, CA, United States).

The raw reads obtained are publicly available at the Sequence Read Archive (SRA) under the accession number: PRJNA701813.

Bioinformatic Analysis

Sequencing data analysis was performed using DADA2 1.14.0 (Callahan et al., 2016) running on R 3.6.2 (R Core Team, 2021). The forward and reverse reads were trimmed to remove low-quality nucleotides and primers sequences using the filterAndTrim function with the following parameters: truncLen = c(290, 220), trimLeft = c(50, 55), and maxN = 0, truncQ = 2. The amplicon sequence variants were inferred using the DADA2 core sample inference algorithm with default parameters. Forward and reverse reads were merged and reads with mismatches were removed. Chimeras were identified using the removeBimeraDenovo function and removed. Further, the SILVA database release 132 (Quast et al., 2013) was used for the taxonomic assignment. Finally, the AVSs table was rarefied to 25,181 reads per sample.

Statistical Analysis

Statistical analyses were carried out using Phyloseq 1.32 (McMurdie and Holmes, 2013) and Vegan 2.5 (Dixon, 2003) packages. The differences between the geophagic and non-geophagic control soil composition were tested via Mann–Whitney *U*-test. Alpha diversity was explored considering the Shannon index and Observed richness calculated from the rarefied AVSs table (25,181 reads). Both indices values were checked for normality using the Shapiro–Wilk test. The possible effects of sex, age class, and family group on alpha diversity indices were evaluated with a Linear Model (ANOVA type III). Beta dispersion was calculated to test if the groups, classified

FIGURE 2 | Visualization of **(A)** a research guide recording the location of a geophagic site (GPS waypoint); **(B)** an indri (*Indri indri*) performing geophagic behavior, eating soil in a specific site; **(C)** a geophagic site under a fallen tree; **(D)** soil horizon eaten in the geophagic site; **(E)** a control site with the upper surface untouched; and **(F)** enlargement of the soil sampled in the control site, under the surface, in the horizon normally eaten by indri. Soil is collected free of debris (grass, leaves, stones, roots).

according to sex, age class, and family group, had the same centroids and heterogeneity. Permutational multivariate analysis of variance (PERMANOVA) was applied to test the possible effect of sex, age class, and family group on the bacterial communities. In addition, the Constrained Analysis of Principal Coordinates (CAP) based on Bray Curtis was used to generate the ordination plots.

Linear discriminant analysis effect size (LEfSe) algorithm (LDA score ≥ 2 and p-value < 0.05) was applied to detect the biomarker taxa for each category (Segata et al., 2011). We excluded from the LEfSe analysis the family groups with less than three individuals (i.e., 4MZ and 6MZ).

RESULTS

Geophagy Site Characterization

Indris were observed to eat soil in sites at the bases of trees uprooted by wind and/or by rainfall, with the lower soil horizons exposed (**Figure 2** and **Supplementary Video 1**). Geophagic and non-geophagic soil samples were characterized by an acidic pH and rich content in total C and N. With regards to the pseudo-total metals, soil samples showed poor content in Calcium (Ca), Phosphorus (P), Sulfur (S), and higher content in Iron (Fe). Manganese (Mn) and Fe were the most extractable in ammonium nitrate in the case of available metals (**Supplementary Table 1**).

Some differences were found between geophagic and non-geophagic sites. Specifically, the concentration of Ca, S, sodium (Na), chromium (Cr), boron (B), and available Nickel (Ni) resulted in being higher in non-geophagic than in geophagic soil samples (p-value < 0.01) (**Figure 3**). On the other hand, for all the other parameters, including pH, total C, total N, the remaining pseudo-total elements, and metals extractable in ammonium nitrate, no statistically significant differences were observed (**Supplementary Table 1**).

Bacterial Taxonomic Community Composition

After quality checking and filtering, 645,297 reads (including non-bacterial reads) were generated from the MiSeq run. The reads assigned as Bacteria were 616,269 resulting in 131 amplicon sequence variants (**Supplementary Tables 2, 3**).

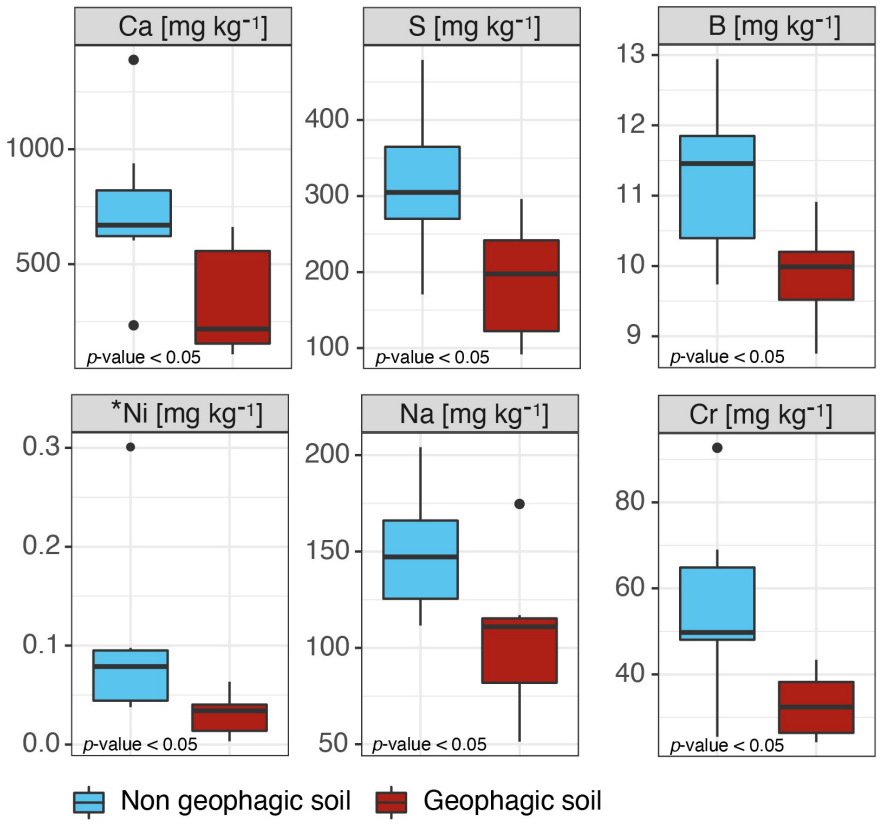

FIGURE 3 | Boxplots representing the chemical parameters resulted statistically different (p-value < 0.05) between the geophagic and non-geophagic soils. *Available metal.

Rarefaction curves showed that all the samples nearly reached the plateau (**Supplementary Figure 1**). All the samples were identified at phylum level: Proteobacteria 40.1 ± 9.5%, Bacteroidetes 28.7 ± 2.8%, Synergistetes 16.7 ± 4.5%, Firmicutes 11.1 ± 1.9%, Verrucomicrobia 2.0 ± 1.2%, Actinobacteria 1.2 ± 0.6%, and Cyanobacteria 0.2 ± 0.3% (**Figure 4A**). At family level the most abundant groups were: Succinivibrionaceae 39.6 ± 11.6%, Prevotellaceae 26.4 ± 3.2%, Synergistaceae 16.7 ± 4.5%, Ruminococcaceae 6.6 ± 2.7%, Acidaminococcaceae 3.3 ± 1.2%, and Puniceicoccaceae 2.0 ± 1.2% (**Figure 4B**). At a finer taxonomic level, the prevalent genera identified were: *Anaerobiospirillum* 39.3 ± 11.9% and Prevotellaceae NK3B31 group 19.8 ± 3.8%, *Cloacibacillus* 8.2% ± 7.2%, *Ruminococcus* 1, 5.0 ± 2.8%, *Jonquetella* 4.24% ± 2.8%, *Pyramidobacter* 4.0 ± 2.8%, *Phascolarctobacterium* 2.6 ± 1.2%, and *Cerasicoccus* 2.0 ± 1.2% (**Figure 4C**).

Effect of Family Group, Sex, and Age Class on Indri Bacterial Diversity

Considering all the individuals, the mean Shannon diversity was 2.61 ± 0.26, whereas the Observed richness's value was 45 ± 7. The values for each individual are reported in **Table 1**. Shannon diversity and Observed richness data resulted to be normally distributed (Shapiro–Wilk normality test: Observed richness, $W = 0.92$, p-value = 0.14; Shannon diversity, $W = 0.91$, p-value = 0.07).

The Linear Model revealed that Observed richness was influenced by family group ($F = 17.69$, p-value = 0.0002), whereas Shannon diversity was affected by both family group ($F = 4.37$, p-value = 0.02) and sex ($F = 10.02$, p-value = 0.01). In particular, females showed higher alpha diversity values if compared to males. Finally, no significant effect was detected according to the age class (**Supplementary Table 4**).

Beta-dispersion of bacterial communities revealed that the samples had homogeneous dispersion (Sex, $F = 1.24$ and p-value = 0.31; family group, $F = 1.21$ and p-value = 0.43; age class $F = 0.002$ and p-value = 0.98). PERMANOVA analysis showed that sex ($F = 7.43$, p-value = 0.001) and family group ($F = 7.4707$, p-value = 0.001) resulted to significantly affect the bacterial communities's beta-diversity, differently from age class ($F = 0.89$, p-value = 0.51). Further, CAP analysis, confirming the results obtained with the PERMANONVA, found that among all the tested possible drivers, sex, and family group influenced the bacterial community's structure (com ∼ family group + Sex; $F = 5.94$ p-value = 0.001) (**Figure 5**).

Linear discriminant analysis effect size algorithm found 15 ASVs biomarkers for the group 1MZ, 17 ASVs with 2MZ, 11 ASVs with 3MZ, and 25 with 8MZ (**Supplementary Table 5**). At phylotype level, Proteobacteria, mainly with the

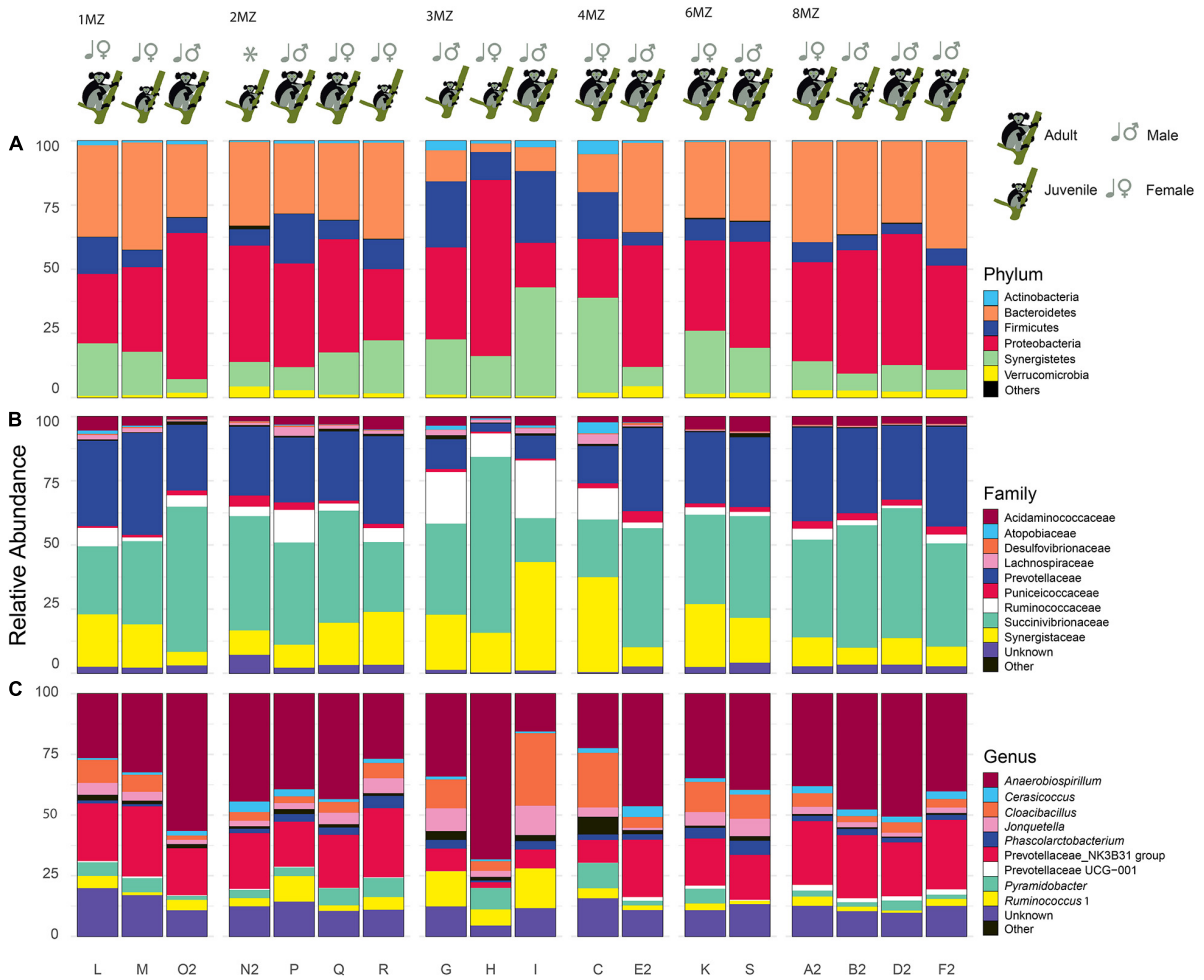

FIGURE 4 | Bar plots of each individual representing the most abundant taxa (average > 1%). Phyum **(A)**, family **(B)**, and genus **(C)**. "Unclassified" represents ASV not classified for the considered taxonomic level. The taxa with a relative average abundance < 1% are collapsed in "Others". In addition, information regarding family group, class age, and sex is reported on the top of each plot.

genus *Desulfovibrio*, characterized the group 2MZ, whereas Actinobacteria with *Atopobium* and Firmicutes with *Tyzzerella* 3 were biomarkers of 3MZ (**Figure 6A**). Further, Bacteroidetes with Prevotellaceae UCG001 and Verrucomicrobia with *Cerasicoccus* were more abundant in the group 8MZ (**Figure 6A**).

Concerning sex, four AVSs biomarkers were found for females and two AVSs males (**Supplementary Table 6**). Moreover, Firmicutes and Synergistetes with the genera *Cloacibacillus* and *Jonquetella* were more abundant in females; differently, Verrucomicrobia with the genus *Cerasicoccus* and Proteobacteria with the genus *Anaerobiospirillum* were mainly present in males (**Figure 6B**).

DISCUSSION

Indris Gut Microbiome Diversity

Although in different proportions, the most abundant phyla found in indris' gut (i.e., Proteobacteria, Bacteroides, and Firmicutes) are consistent with those found in other studies involving primates (Aivelo et al., 2016). On the other hand, the relative abundance of Proteobacteria found in our study was almost five times higher than that found in other lemurs species, such as *Lemur catta* (Umanets et al., 2018), *Eulemur rufifrons*, and *E. rubriventer* (Bennett et al., 2016; **Table 2**). Nevertheless, Greene et al. (2020) investigating wild indris' gut microbiome diversity found a higher abundance of Proteobacteria compared to the other three lemur species (i.e., *L. catta*, *E. rufifrons*, and *E. rubriventer*) (Bennett et al., 2016; Umanets et al., 2018), but still lower than what we found in our work (**Table 2**). With this regard, the high relative abundance of Proteobacteria present in our samples and found in Greene et al. (2020) could represent the typical composition of the gut microbiome of healthy individuals. Differently, in humans, an increased prevalence of Proteobacteria has been observed as a potential signature of dysbiosis (Illiano et al., 2020). Specifically, altered homeostasis, caused by environmental or host factors, such as a low-fiber diet and acute or chronic inflammation, could be a selection driver

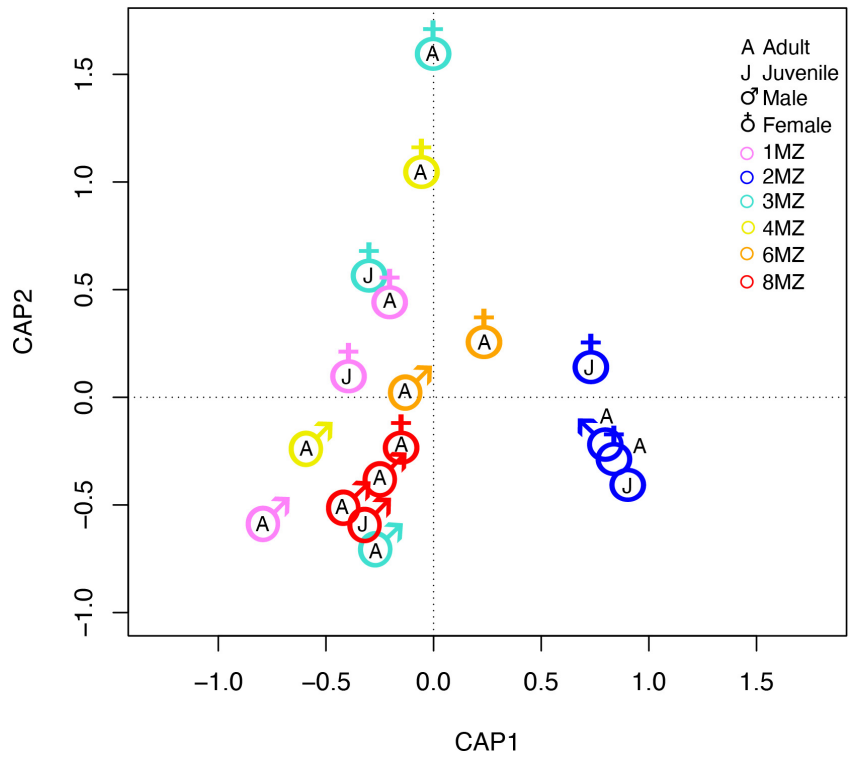

FIGURE 5 | Constrained analysis of principal coordinate ordination plot on bacterial communities of indris fecal samples.

and cause dysbiosis with an increased number of Proteobacteria in the gut. For what concerns the indris, their diet is based on fiber due to its folivores' habitus, with usual consumption of soil as integration. Plant leaves and soil could most likely be an important source of Proteobacteria; in fact, plant leaves, and soil contain about 62 and 36.5% of Proteobacteria, respectively (Shin et al., 2015). Proteobacteria could play a key role in cinnamates degradation and hydroxycinnamates and hydroxycinnamic acids utilization for energy recovery (Greene et al., 2020). Further, indris might rely primarily on Proteobacteria, and secondly on Bacteroidetes and Firmicutes (e.g., *Prevotella* and *Ruminobacter*) for fiber digestion (Biddle et al., 2013). Indeed, Firmicutes members such as Lachnospiraceae and Ruminococcaceae, with some Bacteroidetes, have known fiber fermenting abilities. Interestingly, they have been associated with the production of the appreciated colonocyte nutrient butyrate (Biddle et al., 2013; Meehan and Beiko, 2014). The presence of functionally redundant taxa might support functional stability during ordinary life and possible life disturbance (Vital et al., 2017).

Regarding the factors driving microbial diversity, this study showed the crucial role of social groups in shaping the indris microbiome for the first time. Differences among social groups may be related to feeding and social interactions like grooming, which provide close contact between subjects of the same group (Bennett et al., 2016; Raulo et al., 2018). These mechanisms were identified as relevant factors influencing the microbiome composition of baboons and chimpanzees (Degnan et al., 2012; Tung et al., 2015). A study that analyzed the dynamics of the composition of 10 wild groups in the Maromizaha NAP, comprising the groups sampled in this work, found evidence of only one immigrant female and one immigrant male out of 68 indris over 12 years (Rolle et al., 2021 in press). This very low rate of intergroup mobility limits the number of social partners that indri can have in their lives and, consequently, the intergroup transmission of microorganisms and parasites. In addition, sex was another factor that significantly influenced the microbiome alpha and beta-diversity. Particularly, the higher bacterial Shannon diversity found in females than males could be due to the sex hormones that play a crucial role in sex dimorphism (Haro et al., 2016). Moreover, females showed a higher abundance of *Cloacibacillus* and *Jonquetella*, both belonging to the novel phylum Synergistetes, that inhabits the mammalian gastrointestinal tract typically (Jumas-Bilak et al., 2007; Looft et al., 2013). Differently, males had a higher abundance of bacteria from the *Anaerobiospirillum* genus. This difference can be explained by the fact that females and males differ in nutritional and energetic demands for growth, development, and reproduction. Moreover, sex-specific traits influence the ecological structure of the gut microbiome, maintaining sex differences in physiology and behavior throughout life (Jašarević et al., 2016).

Geophagy in Indris

Typical Oxisols with a reddish color characterized geophagic and non-geophagic sampling sites. Some inherent characteristics of the Oxisols, such as the quite acidic pH, the richness of

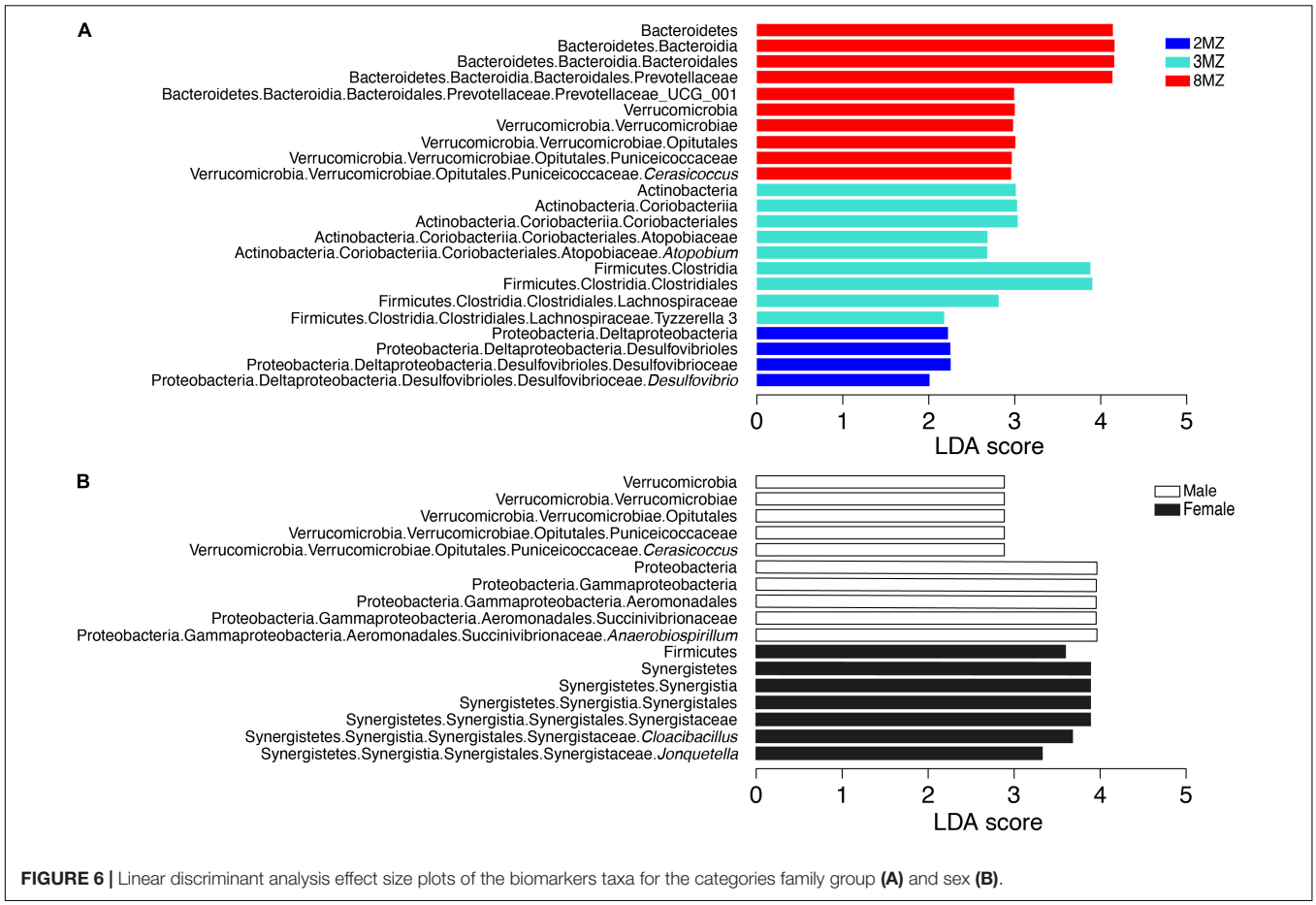

FIGURE 6 | Linear discriminant analysis effect size plots of the biomarkers taxa for the categories family group **(A)** and sex **(B)**.

TABLE 2 | Percentage of the three top bacterial phyla found in this study and other studies.

Lemurs species	References	Firmicutes (%)	Bacteroidetes (%)	Proteobacteria (%)
Lemur catta	Umanets et al., 2018	51.57 ± 0.11	15.81 ± 0.11	5.21 ± 0.11
Eulemur rufifrons and *E. rubriventer*	Bennett et al., 2016	43.3 ± 0.064	30.3 ± 0.053	7.4 ± 0.031
I. indri	Greene et al., 2020	19.70	47.70	20.50
I. indri	This study	11.1 ± 1.9	28.7 ± 2.8	40.1 ± 9.5

secondary oxide-hydroxides and highly weathered clays, seem more important for geophagy than the content in pseudo-total or available elements (Vågen et al., 2006; Borruso et al., 2021). According to the adaptive hypothesis of geophagy, the soil ingested by indri could play a crucial role in micronutrient supplementation and detoxification (i.e., adsorption functions via oxyhydroxides and clays) (Pebsworth et al., 2019). Indeed, indris are folivorous, consuming mainly immature leaves rich in potentially toxic compounds such as tannins, terpenes, and cyanogenic glycosides derived (Hemingway, 1998); thus, the geophagic soil could be involved in the plant's toxin adsorption derived from the diet (de Souza et al., 2002; Pebsworth et al., 2019).

However, the reason behind the selection of one site instead of another one remains unclear. The choice of the sites characterized by the exposition of lower soil horizons could be a strategy to limit the energy expended in obtaining soil from the intact ground. Nevertheless, some elements (i.e., Ca, S, Na, Cr, B, and available Ni) were present at lower concentrations in geophagic than in non-geophagic soil. Although we cannot directly explain these differences, they could indicate that other soil quality traits could orientate the selection of a specific soil.

In conclusion, studies on different species suggested that geophagic sites are required to maintain individual and population health (Pebsworth et al., 2019). Accordingly, preserving the geophagic sites is crucial in wildlife conservation policy.

Microbial Ecology and Indri Conservation

Microbial ecology offers valuable perspectives to investigate primate health and improve conservation efforts. Understanding the drivers affecting the microbiome associated with the host (e.g., indri) is critical for conservation biology. It is well known that the microbial gut communities profoundly affect host health,

nutrition, physiology, and immune systems (Sandri et al., 2020). For instance, our study is fundamental to document the typical composition of healthy individuals considering sex and group influence (Amato et al., 2020). Therefore, many studies have been conducted on the human microbiome where microbial biomarkers of health have been shown, such as the presence of *Faecalibacterium prausnitzii* (Manor et al., 2020). The acquisition of new information about animal gut microbiomes can help identify biomarkers for animal health. In addition, microbial gut communities are sensitive to environmental alterations and their diversity seems to be correlated with habitat quality and, thus, with possible health implications (Scotti et al., 2017). The application of gut microbiome analyses to wildlife conservation of endangered species is currently in its infancy but holds enormous potential. To date, no conservation policy or legislation includes microbiome assessments. Integrating a new understanding of the patterns of microbial diversity and early signs of impending microbial disruption offer valuable tools for informing conservation strategies and monitoring and promoting primate health (Stumpf et al., 2016). The present study represents a first insight toward understanding the overall diversity and ecology of indris microbiome in different familiar groups and a sex-dependent baseline that can be tracked over time as a component of efforts to help animal conservation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Sequence Read Archive (SRA) BioProject ID: PRJNA701813.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because. The non-invasive methods used for fecal collections of wild indris adhere to the International Primatological Society (IPS) "Principles for the Ethical Treatment of Non-Human Primates." Field data collection protocols were reviewed and approved by Madagascar's Ministere de l'Environnement, de l'Ecologie et des Forêts (Permit 2018: N° 91/18/MEEF/SG/DGF/DSAP/SCB.Re). Field data collection protocols were also approved by Groupe d'Étude et de Recherche sur les Primates de Madagascar (GERP), the association governing research in the Maromizaha New Protected Area.

AUTHOR CONTRIBUTIONS

CSa CSp, CG, PM, PT, and LB conceived and designed the experiments. VT and RR supervised the collection of the fecal and soil samples. FC, AC, DL, LC, and MM carried out the experiments. FC, LB, DL, MM, VT, LC, MS, TM, AC, MD, and FB analyzed the data. FC, AC, LB, CSa, CSp, CG, VT, SC, TM, and PM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.668274/full#supplementary-material

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Disentangling the possible drivers of *Indri indri* microbiome: A threatened lemur species of Madagascar

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Contents

1. SUPPLEMENTARY TABLES

Table S1. Geophagic and non-geophagic soil characteristics (average ± standard error) and Mann–Whitney test *p*-value (n.s. = not significant for *p*-value > 0.05, DL = detection limit)

Parameter	Unit	Non-geophagic soil	Geophagic soil	*p*-value
pH		4.17 ± 0.14	4.51 ± 0.13	n.s.
Total N	%	0.17 ± 0.03	0.22 ± 0.04	n.s.
Total C	%	2.40 ± 0.42	3.14 ± 0.54	n.s.
C/N ratio		14.3± 0.6	14.1 ± 0.7	n.s.
Pseudo total elements:				
Al	mg kg^{-1}	72200 ± 3280	71300 ± 6870	n.s.
As	mg kg^{-1}	0.346 ± 0.223	1.001 ± 0.392	n.s
B	mg kg^{-1}	11.2 ± 0.4	9.8 ± 0.3	0.03
Ba	mg kg^{-1}	18.5 ± 4.6	28.0 ± 8.6	n.s.
Be	mg kg^{-1}	0.615 ± 0.164	0.760 ± 0.148	n.s.
Ca	mg kg^{-1}	739 ± 133	344 ± 89	0.01
Co	mg kg^{-1}	4.70 ± 1.18	5.85 ± 1.49	n.s
Cr	mg kg^{-1}	56.2 ± 7.9	32.7 ± 2.8	0.02
Cu	mg kg^{-1}	12.5 ± 4.7	9.9 ± 2.1	n.s
Fe	g kg^{-1}	28.7 ± 2.6	32.4 ± 5.4	n.s
K	mg kg^{-1}	670 ± 219	840 ± 308	n.s
Li	mg kg^{-1}	3.63 ± 1.11	4.85 ± 1.52	n.s
Mg	mg kg^{-1}	353 ± 113	267 ± 75	n.s
Mn	mg kg^{-1}	120 ± 37	199 ± 54	n.s
Mo	mg kg^{-1}	2.86 ± 0.59	2.66 ± 0.57	n.s
Na	mg kg^{-1}	149 ± 12	104 ± 15	0.04
Ni	mg kg^{-1}	15.70 ± 5.92	10.71 ± 3.55	n.s
P	mg kg^{-1}	229 ± 34	243 ± 41	n.s
Pb	mg kg^{-1}	34.1 ± 3.1	49.4 ± 13.1	n.s
S	mg kg^{-1}	317 ± 37	187 ± 30	0.02
Si	mg kg^{-1}	302 ± 147	300 ± 93	n.s
Sn	mg kg^{-1}	2.70 ± 0.36	2.96 ± 0.34	n.s
Sr	mg kg^{-1}	10.67 ± 2.09	5.43 ± 1.12	n.s
Ti	mg kg^{-1}	1771 ± 139	1804 ± 398	n.s
V	mg kg^{-1}	34.4 ± 9.9	41.5 ± 19.7	n.s
Zn	mg kg^{-1}	54.9 ± 7.6	57.9 ± 10.3	n.s
Available metals:				
Cd	mg kg^{-1}	< DL	< DL	n.s
Co	mg kg^{-1}	< DL	< DL	n.s
Cr	mg kg^{-1}	< DL	< DL	n.s
Cu	mg kg^{-1}	0.023 ± 0.010	0.022 ± 0.008	n.s
Fe	mg kg^{-1}	8.64 ± 4.13	6.00 ± 2.27	n.s

Mn	mg kg^{-1}	8.91 ± 3.73	3.40 ± 1.28	n.s
Ni	mg kg^{-1}	0.099 ± 0.03	0.030 ± 0.011	0.015
Pb	mg kg^{-1}	0.584 ± 0.138	3.167 ± 1.197	n.s
Zn	mg kg^{-1}	0.712 ± 0.221	0.425 ± 0.161	n.s

Table S2: Bacterial sequences obtained per each sample after filtering.

Sample	Bacterial reads
A2	48,119
B2	36,307
C	27,177
D2	38,312
E2	28,294
F2	45,553
G	26,102
H	34,972
I	27,262
K	37,193
L	39,381
M	25,181
N2	31,,619
O2	34,686
P	32,653
Q	32,819
R	35,349
S	35,242

Table S3: Statistic summary of the overall bacterial sequences.

Bacterial reads summary	
Total count	616,221
Min	25,181
Max	48,119
Median	34,829
Mean	34,235
Std. dev.	6,293

Table S4. Linear model of the Observed richness and Shannon indices tested for the categories: family group, sex and age.

Alpha diversity linear model					
Observed richenss	Sum. Sq.	Df	F value	*p*-value	
(Intercept)	4249.0	1	496.34	3.499e-09	***
Group	757.3	5	17.69	0.0002	***
Sex	0.4	1	0.04	0.84	
Class age	0.2	1	0.02	0.88	
Residuals	77.0	9			
Shannon	Sum. Sq.	Df	F value	*p*-value	
(Intercept)	15.8816	1	488.58	3.751e-09	***
Group	0.7111	5	4.37	0.02	*
Sex	0.3259	1	10.02	0.01	*
Class age	0.0026	1	0.08	0.78	
Residuals	0.2925	9			

Table S5. LEfSe analysis identification of the biomarker taxa for the category family groups.

Taxonomy		LDA	*p*-value
Bacteria.Actinobacteria	3MZ	3.01	0.0186
Bacteria.Actinobacteria.Coriobacteriia	3MZ	3.03	0.0186
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales	3MZ	3.03	0.0186
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Atopobiaceae	3MZ	2.68	0.0112
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Atopobiaceae.*Atopobium*	3MZ	2.68	0.0112
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Atopobiaceae.*Atopobium*.ASV49	3MZ	2.70	0.0112
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.*Collinsella*.ASV390	3MZ	2.33	0.0051
Bacteria.Bacteroidetes	8MZ	4.14	0.0496
Bacteria.Bacteroidetes.Bacteroidia	8MZ	4.16	0.0496
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales	8MZ	4.16	0.0290
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae	8MZ	4.14	0.0439
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV105	2MZ	2.72	0.0334
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV106	8MZ	2.74	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV11	1MZ	3.57	0.0155
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV12	2MZ	3.76	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV13	8MZ	3.63	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV14	2MZ	3.47	0.0271
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV18	8MZ	3.45	0.0101
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV213	8MZ	2.41	0.0334
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV242	2MZ	2.44	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV264	8MZ	2.39	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV280	8MZ	2.30	0.0167
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV4	1MZ	3.87	0.0072
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV52	3MZ	3.08	0.0482

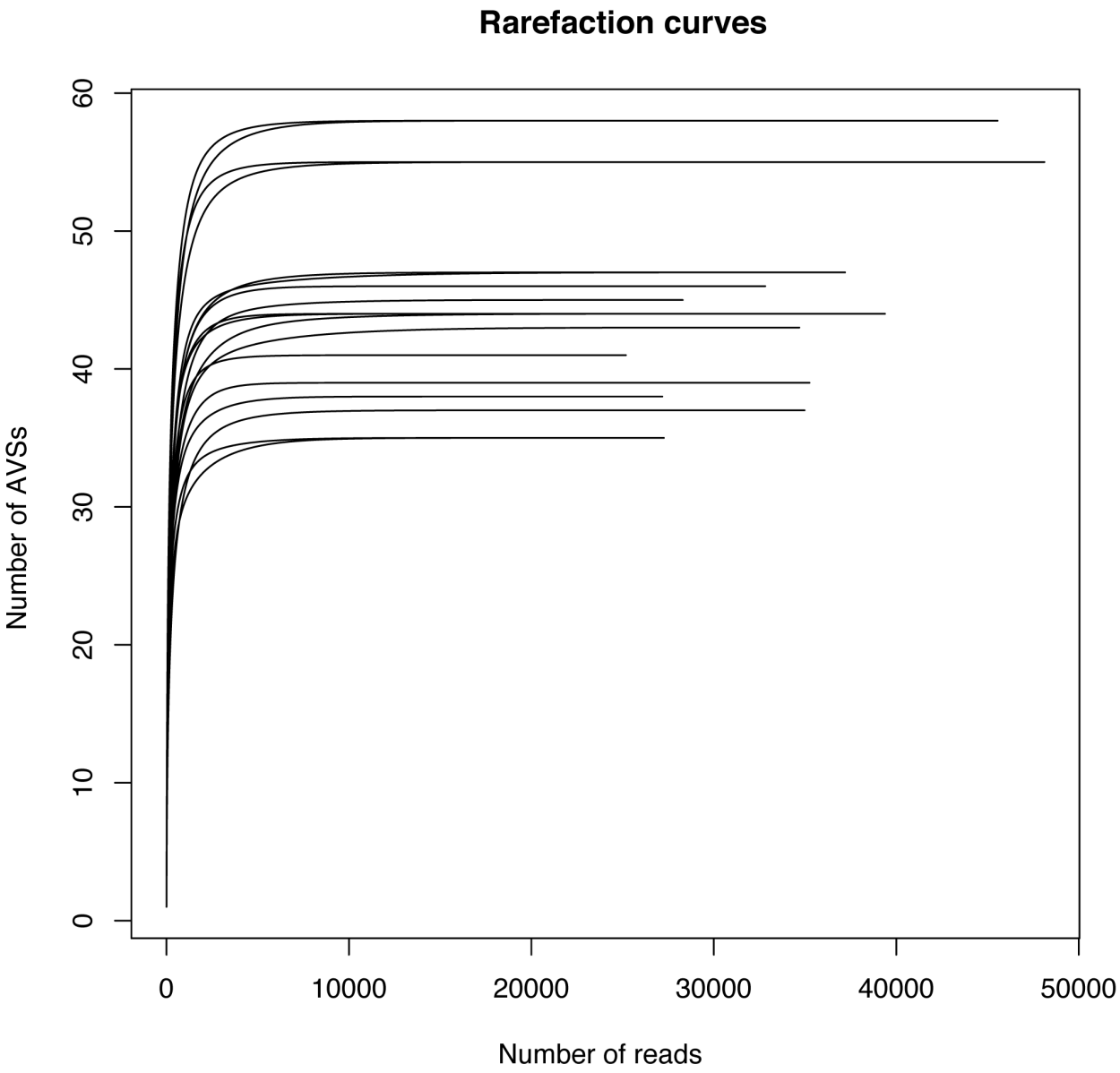
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV56	8MZ	2.83	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group.ASV674	8MZ	2.01	0.0334
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001	8MZ	2.99	0.0091
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001.ASV173	1MZ	2.40	0.0082
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001.ASV182	8MZ	2.36	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001.ASV246	8MZ	2.39	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001.ASV541	8MZ	2.13	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001.ASV59	8MZ	2.61	0.0072
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV15	1MZ	3.35	0.0082
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV167	1MZ	2.65	0.0051
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV20	2MZ	3.41	0.0095
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV311	1MZ	2.47	0.0051
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV33	8MZ	3.05	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV36	1MZ	3.06	0.0070
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV371	2MZ	2.08	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV412	1MZ	2.35	0.0051
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.ASV74	8MZ	2.48	0.0081
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV1127	1MZ	2.47	0.0051
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV146	2MZ	2.64	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV175	2MZ	2.58	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV204	3MZ	2.01	0.0482
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV248	8MZ	2.50	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV316	8MZ	2.36	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV350	8MZ	2.22	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV351	1MZ	2.46	0.0051
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV359	8MZ	2.27	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV363	8MZ	2.37	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV393	8MZ	2.41	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV394	8MZ	2.59	0.0057
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV46	1MZ	2.75	0.0106
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.ASV474	1MZ	2.16	0.0051

Table S6. LEfSe analysis identification of the biomarker taxa for the category sex.

Taxonomy	Gender	LDA	p value
Synergistetes.Synergistia.Synergistales.Synergistaceae.*Jonquetella*	Female	3.32	0.0053
Synergistetes.Synergistia.Synergistales.Synergistaceae	Female	3.89	0.0021
Synergistetes.Synergistia.Synergistales	Female	3.89	0.0021
Synergistetes.Synergistia.Synergistales.Synergistaceae.*Cloacibacillus*.ASV3	Female	3.67	0.0029
Firmicutes	Female	3.59	0.0269
Synergistetes	Female	3.89	0.0021
Synergistetes.Synergistia.Synergistales.Synergistaceae.*Jonquetella*.ASV7	Female	3.31	0.0124
Synergistetes.Synergistia.Synergistales.Synergistaceae.ASV130	Female	3.71	0.0021

Synergistetes.Synergistia	Female	3.89	0.0021
Synergistetes.Synergistia.Synergistales.Synergistaceae.Cloacibacillus	Female	3.68	0.0039
Actinobacteria.Coriobacteriia.Coriobacteriales.ASV331	Female	3.41	0.0427
Verrucomicrobia.Verrucomicrobiae.Opitutales.Puniceicoccaceae.*Cerasicoccus*	Male	2.89	0.0343
Proteobacteria.Gammaproteobacteria	Male	3.96	0.0015
Verrucomicrobia	Male	2.89	0.0343
Proteobacteria.Gammaproteobacteria.Aeromonadales.Succinivibrionaceae.Anaerobiospirillum	Male	3.96	0.0015
Proteobacteria	Male	3.96	0.0015
Verrucomicrobia.Verrucomicrobiae.Opitutales.Puniceicoccaceae	Male	2.89	0.0343
Verrucomicrobia.Verrucomicrobiae	Male	2.89	0.0343
Proteobacteria.Gammaproteobacteria.Aeromonadales.Succinivibrionaceae	Male	3.95	0.0015
Verrucomicrobia.Verrucomicrobiae.Opitutales.Puniceicoccaceae.*Cerasicoccus*.ASV16	Male	2.89	0.0433
Proteobacteria.Gammaproteobacteria.Aeromonadales.Succinivibrionaceae.*Anaerobiospirillum*.ASV1	Male	3.92	0.0206
Verrucomicrobia.Verrucomicrobiae.Opitutales	Male	2.89	0.0343
Proteobacteria.Gammaproteobacteria.Aeromonadales	Male	3.95	0.0015

Figure S1. Rarefaction curves of the indris' fecal samples



4 The mycobiota of *Indri indri* and the relationship with the soil.

Most of the studies focus on gut bacteria; however, archaea, virus and also fungi are components of the gut microbiota. In particular, fungi serve essential functions in gut homeostasis. Fungi are relevant and relatively neglected taxa contributing to host immunity and gut health and are possibly associated with disease susceptibility (for a review, see Enaud et al., 2018). The fungal community - the mycobiota - in wild animals are less explored (Strati et al., 2016; Nam et al., 2008), but the composition and diversity of plant-degrading fungi in the gut seem to be linked with phylogeny in herbivorous mammals (Hager and Ghannoum, 2017; Liggenstoffer et al., 2010). Gut fungi have also recently been shown to vary with sex, age, and season in a group of macaques (Sun et al. 2018). Moreover, a study investigates bacteria and fungi communities in two different primate species with different ecology and found differences in fungi more than in bacteria between the gut of the two species. Considering the central role that fungi in maintaining intestinal homeostasis and systemic immunity (Iliev and Leonardi, 2017), variation in their diversity may impair host health and gut homeostasis. Thus, investigating the fungal microorganisms in the gut become crucial for the conservation of animal species, especially for those that face constant threat due to habitat modification such as primate hosts. Although still poorly understood, the different dietary habits may drive diversification of fungal communities across species. For a folivores species that use also the soil as integration part of the diet to analyze the fungi composition of soil and gut could be extremely important. However, the effect of habitat fragmentation and host lifestyle on gut fungi is unknown, as are the interactions between fungal and bacterial microbiota components (Barelli et al., 2020).

The following study provides an insight in the gut mycobiota of *Indri indri* and its similarities and differences with the soil of the area in which the indris live. Since every effort to host the species in controlled environment has been failed, by involving experts in the field of soil and fungi communities, this study deeply explores the importance of the link between the species and the environment.

4.1 The third paper

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I like the way you eat it: Lemur (*Indri indri*) gut mycobiome and geophagy

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FUNGAL MICROBIOLOGY

I Like the Way You Eat It: Lemur (*Indri indri*) Gut Mycobiome and Geophagy

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Abstract

Here, we investigated the possible linkages among geophagy, soil characteristics, and gut mycobiome of indri (*Indri indri*), an endangered lemur species able to survive only in wild conditions. The soil eaten by indri resulted in enriched secondary oxide-hydroxides and clays, together with a high concentration of specific essential micronutrients. This could partially explain the role of the soil in detoxification and as a nutrient supply. Besides, we found that soil subject to geophagy and indris' faeces shared about 8.9% of the fungal OTUs. Also, several genera (e.g. *Fusarium*, *Aspergillus* and *Penicillium*) commonly associated with soil and plant material were found in both geophagic soil and indri samples. On the contrary, some taxa with pathogenic potentials, such as *Cryptococcus,* were only found in indri samples. Further, many saprotrophs and plant-associated fungal taxa were detected in the indri faeces. These fungal species may be involved in the digestion processes of leaves and could have a beneficial role in their health. In conclusion, we found an intimate connection between gut mycobiome and soil, highlighting, once again, the potential consequent impacts on the wider habitat.

Keywords Mycobiome · Gut · Soil quality · Non-human primates · Conservation · *Indri indri*

Introduction

Geophagy, the intentional consumption of soil, is practiced by many different human cultures over different continents [1]. Cultural tradition, together with sensory trap, hunger or stress relief, are the main three non-adaptive explanations for human geophagy [1]. The well-documented occurrence of geophagic behaviour in many vertebrates, including non-human Primates, encourages the formulation of two main adaptive hypotheses: (i) the supplementation function consisting of supplementing microelements that are lacking in the diet and (ii) the protective function of soil in pH regulation, against toxins and parasites [2]. In this respect, lemurs' radiation in more than 100 species, colonizing different habitats and

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performing soil eating in at least 40 species, make them a very promising model for untangling the causes and functional consequences of geophagy [3]. Indri (*Indri indri*), the biggest among living lemurs, has a well-documented feeding and geophagy behaviour. This lemurs species is classified as 'critically endangered' by the IUCN Red List of Threatened Species due to the destruction and fragmentation of its habitat [4]. Furthermore, indris have never been successfully bred in captivity [5]. This evidence suggests that some behavioural and environmental factors are not satisfied with the already tested captivity protocols.

The indris' diet is mainly folivorous (i.e. immature leaves), but it may include even bark, seeds, flowers and fruits [5] (Fig. 1 b, c and d, Supplementary Video S1). In the folivorous diet, the interactions between host and gut microbiome are necessary for the processes involved in cellulase activities due to the absence of these enzymes in all vertebrates [6, 7]. Primates' gut harbour a plethora of microbes, including archaea, bacteria and fungi, which play a crucial role in the digestion process, health and behaviour [8–11]. Despite the limited studies on primate mycobiome, indications are now accumulated on the fungi's important role in host physiology [12, 13]. However, there is evidence that the primates' gut lacks a stable core mycobiome, unlike the bacterial microbiome [14]. In this respect, to define if a fungal species inhabits the gut stably or transiently remains an open question. The high inter-individual variability can be explained considering that diet, geography and environment are the primary drivers in shaping the mycobiome composition [10] and fungal species of environmental or food-associated origin could transiently colonise the gut influencing the mycobiome composition. In this regard, we aimed to investigate the linkages between geophagic soil and indris gut mycobiome, using samples collected in the Maromizaha forest (Madagascar) (Fig. 1a). Specifically, we evaluated (i) the possible role of the soil properties eaten by indris and (ii) the putative geophagic soil contribution to the fungal communities inhabiting the indris' gut.

Material and Methods

Behavioural Observation, Faecal and Soil Sample Collection

All the samples were obtained from indris (faecal material) and geophagic soils in Maromizaha forest (latitude 18° 57′ S and 19° 00′ S, longitude 48° 26′ E and 48° 31′ E, Madagascar) between December 4 and 6, 2018 (Fig. 1a). Indris has been the subject of ongoing etho-ecological studies since 2009 [15]. Records are kept as photographs or videos.

Faecal samples were collected from 9 individuals (Table 1) following the groups' activity patterns in their natural habitat. Every single individual was recognised by natural trough marks [16]. We collected faecal samples immediately after

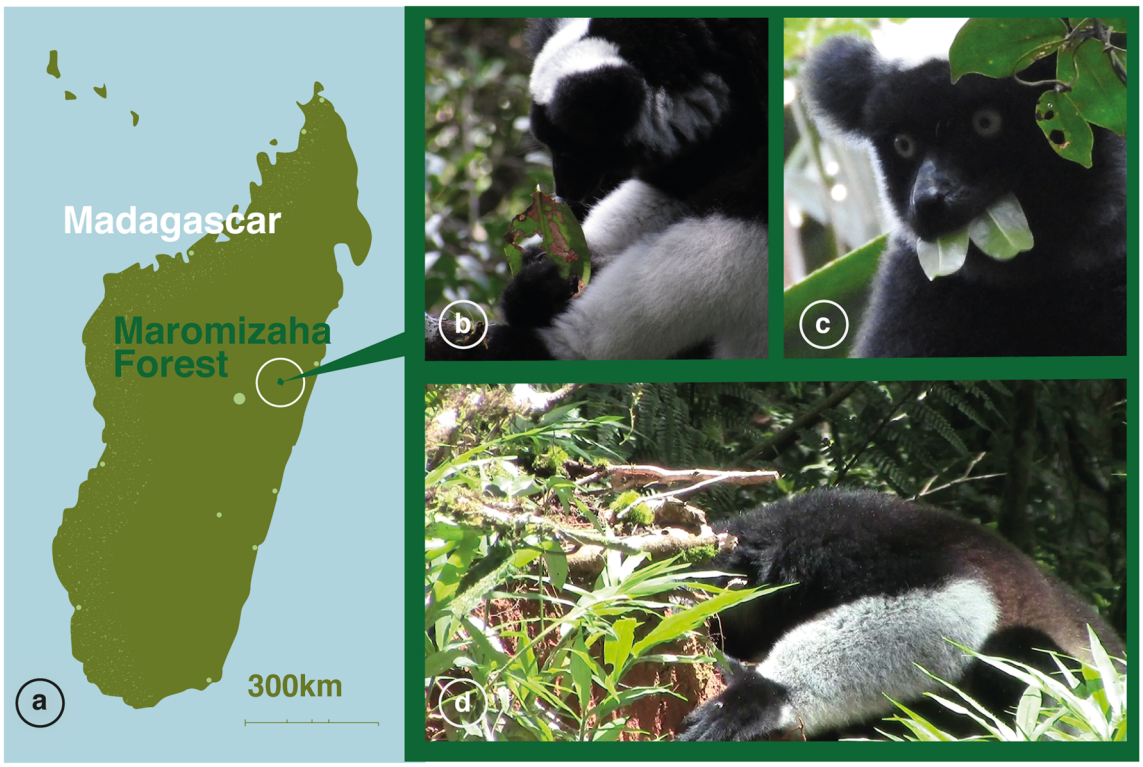

Fig. 1 a Localisation of the study site, the Maromizaha Forest, in north-eastern Madagascar; (**b**) indri eating a mature, speckled leaf; (**c**) indri eating young leaves; (**d**) indri eating soil from a fallen tree site

Table 1 Information of the indris and soils considered

Name	Group	Age (years)	Sex	Geophagic behaviour
Bemasoandro	8MZ	> 6	Female	Yes
Emè	8MZ	1	Male	Yes
Zafy	8MZ	6	Male	Yes
Eva	4MZ	> 6	Female	Yes
Koto	4MZ	> 6	Male	Yes
Mahagaga	3MZ	> 6	Male	Yes
Bevolo	1MZ	> 6	Female	Yes
Cami	1MZ	1	Female	Yes
Dary	2MZ	6 months	Unknown	Yes

defecation, when only one animal was present, to avoid individual misidentification during the sampling process [15]. Disposable sterile gloves were worn when collecting samples to prevent contamination. Specifically, about 5 g of faeces was collected into screw-capped tubes, with an integrated plastic shovel-like tool attached to the cap, containing 10 ml of RNAlater (Thermofisher, Waltham, MA, USA). The stool amount was taken from the middle of each fresh piece of faeces to avoid soil contamination. Next, the small plastic shovel-like tool attached to the cap of screw-capped tubes was used to scoop faecal samples. Every container was sealed immediately after the collection to avoid cross-contamination among samples. Seven soil samples (Table 1) were collected from the seven geophagy sites. All samples have been preserved in a portable refrigerator and then stored at − 20 °C in the laboratory until downstream analysis.

Soil Characterisation

Soil samples were air-dried, milled and sieved at 2 mm for soil analysis in agreement with SSSA methods [17]. Briefly, total carbon (C_{tot}) and total nitrogen (N_{tot}) were determined using an elemental analyser (Flash 2000, Thermo Scientific, Germany) coupled with an isotopic mass spectrometer (DELTA Advantage, Thermo Scientific, Germany). Pseudo total element concentration was determined after acid mineralisation with aqua regia and hydrogen peroxide in an Ethos TC microwave lab station (Milestone, Bergamo, Italy) by an inductively coupled plasma optical emission spectrometer (ICP-OES, Ametek Spectro, Arcos, Germany). Iron, aluminium, titanium and silica oxide concentrations were determined by ICP-OES (Ametek Spectro, Arcos, Germany) after extraction with sodium dithionite ($Na_2S_2O_4$).

DNA Extraction and NGS Sequencing

Total DNA extraction from 200 mg faecal and soil samples was carried out using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) with a modification to the protocol including a pre-treatment with lyticase. Briefly, the samples were initially treated with 200 U lyticase (Sigma-Aldrich Co., Gillingham, UK), homogenised and incubated for 30 min at room temperature [18]. Lastly, the DNA was eluted twice to improve yield. Extracted DNA was quantified using a QuBit 2.0 Fluorometer Assay (Life Technologies Corporation) and then adjusted at 1 ng μL^{-1}.

Fungal ITS region was amplified using the primer pairs ITS3 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC 3′) modified with the required Illumina sequencing adaptors [19]. PCR was conducted in a total reaction volume of 25 μl using the Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy), 1 μl of each primer (10 μM) and 2.5 μL of DNA template. In all samples, 0.4 mg/ml BSA was added. The thermal cycling protocol consisted of 94 °C for 2 min followed by 30 cycles each of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C and final elongation at 72 °C for 5 min. The libraries were prepared by BMR-Genomics Ltd. (http://www.bmr-genomics.it/) and sequenced on the MiSeq platform (Illumina Inc., San Diego, Ca, USA).

Bioinformatics Analysis and Statistical Analysis

Raw data were quality checked via FastQC [20]. Sequences were pre-processed, quality filtered, trimmed, de-noised, merged, modelled and analysed via DADA2 within QIIME2 [21]. Chimeras were discarded using the 'consensus' method [22]. Finally, the sequences variants were clustered using VSEARCH with a cut-off of 97% [23]. The taxonomy annotation was performed using a Naïve-Bayes classifier trained on the UNITE+INSD database against the representative sequences [24]. The taxonomic annotated OTU table was parsed against the FunGuild (v1.0) database to assign putative functional guilds to each sample [25]. All sequences have been submitted to the European Nucleotide Archive (EMBL-EBI) under the study accession number PRJEB39443 (sample accession number from ERS4827963 to ERS4827978). *Cryptococcus* sequences were aligned using CLUSTALW [26]. For phylogenetic reconstruction, the neighbour-joining algorithm and Kimura's two-parameter model were used with complete deletion of positions containing gaps or missing data and 1000 bootstrap replications [27]. Phylogenetic analyses were carried out in MEGAX version 10.2 [27].

Rarefaction curves and Venn diagram were created using 'ggplot' and 'vegan' packages within the 'R' environment [28–30]. Linear discriminant analysis effect size (LEfSe) algorithm (considering an LDA score ≥ 2 and p value < 0.05) was applied to discover the most abundant fungal genera (average > 0.3%) and functional guilds associated with indri and

soil samples [31]. All the analyses were performed on rarefied data to 1154 reads.

Results and Discussion

Geophagy

In all nine individuals considered in this study (Table 1), we observed soil eating behaviour and a quite stereotypical ingestion method (Fig. 1d; Supplemental Video S1). The focal group always moved to a precise location solely for soil consumption. In all soil feeding-bouts observed, an indri descended first to the ground, jumping from a tree or a liana near the geophagy site, and started to eat soil. During one soil feeding-bout, one member of the group began to eat. The other members approached the site and stayed on the nearest trees monitoring the surrounding environment (< 10 min) till he/she left the site, and a new indri took his/her turn in eating soil, one after the other.

The individuals consumed the soil directly by eating the exposed horizons with the mouth or collecting a small amount of soil with the hand and successively introducing it into the mouth.

When the mother carried the babies (i.e. Eme and Cami) (Table 1) and the female entered the site, we observed geophagy also in the youngest animals (Supplemental Video S1). After all individuals had fed, the group scurried out of the geophagy site. Next, the group reached a new location for eating or resting. Geophagy sites observed were mostly in the proximity of fallen trees, landslides or soft mounds of earth, revealing the lower soil horizons. There were exposed soils at the bases of trees uprooted by wind or rainfall in the valley, at lower elevations, in the slopes. All the locations were relatively free of debris (grass, leaves, stones, etc.) (Supplementary Video S1).

Geophagic Soil Composition

Soil composition analysis revealed that the different sampling sites might be classified as Oxisols rich in secondary oxide-hydroxides and highly weathered clays [32]. In particular, soil analysis revealed that the sandy loam was characterised by a quite acid pH, relatively rich in organic carbon, total nitrogen, potassium and magnesium, but poor in phosphorous and calcium (Table 2). Soil components as secondary oxide-hydroxides are characterised by a high specific surface area, being thus ideal candidates for the gut detoxification of indri. This type of soil could be involved in the plant toxin adsorption, such as tannins, terpenes and cyanogenic glycosides derived from the diet based on immature fruits and leaves [2, 33]. Further, the low pH is a common characteristic of geophagic soils [34]; a consequent higher metal availability could be advantageous for their incorporation in the biological processes. In addition, soils were rich in manganese (Mn) and iron (Fe) (Table 2). These essential micronutrients might thereby contribute to both enhanced enzymatic activities and an important nutrient supply playing a crucial role in the indri physiology [35]. Further, heavy metals found in the soil, such as cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni) and zinc (Zn), were suitably below the threshold value for either ecological and health risks (Table 2) [36].

Table 2 Geophagic soil characteristics, average and standard error (es)

Geophagic soil		Average ± es
Granulometry	Clay	14.7 ± 1.4
	Silt	8.9 ± 0.9
	Sand	76.4 ± 1.2
	pH (H2O)	4.2 ± 0.1
Total carbon and nitrogen	N (%)	0.23 ± 0.02
	C (%)	3.18 ± 0.31
	C/N	13.71 ± 0.41
Pseudo total elements (mg/kg)	Al	77273 ± 5638
	Ca	427 ± 91
	Co	6.08 ± 1.61
	Cr	46.67 ± 9.64
	Cu	15.49 ± 4.48
	Fe	39394 ± 6102
	K	476 ± 192
	Mg	263 ± 108
	Mn	201 ± 61
	Mo	2.57 ± 0.58
	Na	104 ± 14
	Ni	17.35 ± 5.81
	P	287 ± 51
	Pb	39.93 ± 5.28
	S	279 ± 16
	Si	279 ± 98
	Sn	3.17 ± 0.28
	Ti	2212 ± 491
	V	67.42 ± 24.54
	Zn	55.51 ± 10.10
Dithionite-extractable metals (mg/kg)	Al	3580 ± 463
	Fe	7988 ± 852
	Ti	156 ± 37

Mycobiome of the Geophagic Soil and Indri

After bioinformatics analysis, we obtained 437,872 reads clustered in 1110 OTUs (97% identity). Rarefaction curves showed that almost all the soil and indris faecal samples nearly reached plateau (Fig. S1). We found that 74 (8.9%) of the OTUs were shared between soil and indris' faeces samples

(Fig. 2). To the best of our knowledge, only another work has investigated the possible overlap between microbial species in the gut and soil [37]. The authors analysed more than 3000 samples, finding a low number of microbial classes shared between soil and gut. In addition, we re-analysed the OTU table of Tasnim et al. [37], and we found a considerably lower percentage (~ 2%) of shared OTUs (i.e. soil and gut) than in our dataset.

Although with differences in relative abundance, some genera were found both in soil and indris' faeces, including *Fusarium*, *Aspergillus*, *Penicillium*, *Apiotrichum*, *Ganoderma*, *Mortierella*, *Metarhizium*, *Tolypocladium* and *Chaetosphaeria* (Fig. 3). Several members affiliated to the genera *Fusarium, Aspergillus* and *Penicillium* have been commonly found in primates, especially with a vegetarian diet, as well as in forest soil and leaves of herbaceous and woody plants [10, 38, 39]. In some species of *Aspergillus* and *Penicillium* is reported the presence of catalytic enzymes such as pectin methyl esterase and polygalacturonase involved in plant polysaccharide degradation [39–41]. Besides, xylanase genes linked with the degradation of xylan, xylose and/or carboxymethyl cellulose have been detected in some *Fusarium* species [39, 42]. *Apiotrichum Mortierella* and *Ganoderma* are soil-associated genera involved in the decomposing of plant material, and some members may be associated with mammals [43–48]. Further, *Chaetosphaeria* is a cosmopolitan genus mainly found in the soil, rhizosphere or plant material [43, 49], and Metarhizium and Tolypocladium are entomopathogenic fungal taxa associated with soil-borne insects [50, 51].

On the contrary, *Candida* and *Cryptococcus* that are frequently detected in human and non-human primates' gastrointestinal tracts were only present in faecal samples [10, 44, 52] (Fig. 3). A few species affiliated to *Cryptococcus* (i.e. *C. neoformans*) can cause Cryptococcosis, an animal-associated infectious disease with a worldwide distribution [53]. Further, these species can grow and proliferate in the decomposing wood of tree holes and the soils covered by plant debris [53–55]. Consequently, the pathogen can be spread among individuals via an environmental or zoophilic way [53, 55]. Although we are aware of the technical limitation (i.e. short reads), the OTU 2157 (with the highest frequency among *Cryptococcus* OTUs) resulted in being the closest relative with *C. neoformans* (Fig. S2). The detection of this taxon could be seen as a health problem for potential overlap with humans, specifically for the rural communities present in the area.

Furthermore, fungal species only present in indris' faeces were *Nigrospora* and *Meyerozyma*, which have been frequently found in association with primates, leaves and soil [47, 56] (Fig. 3).

The high percentage of 'plant-associated' fungi in the indris' mycobiome is not surprising considering the linkages between the folivorous diet and the consequent accumulation of leaf-associated microbes in their gut (Fig. 4). For instance, these environmental fungi may survive, influence and, in some cases, colonise the gut [52]. Yet, the high percentage of 'undefined saprotrophs' fungal species may assist the breakdown of indigestible leaf cellulose and the redistribution of the nutrients [39, 57] (Fig. 4). In soil, saprophytic fungi are well known for the production of

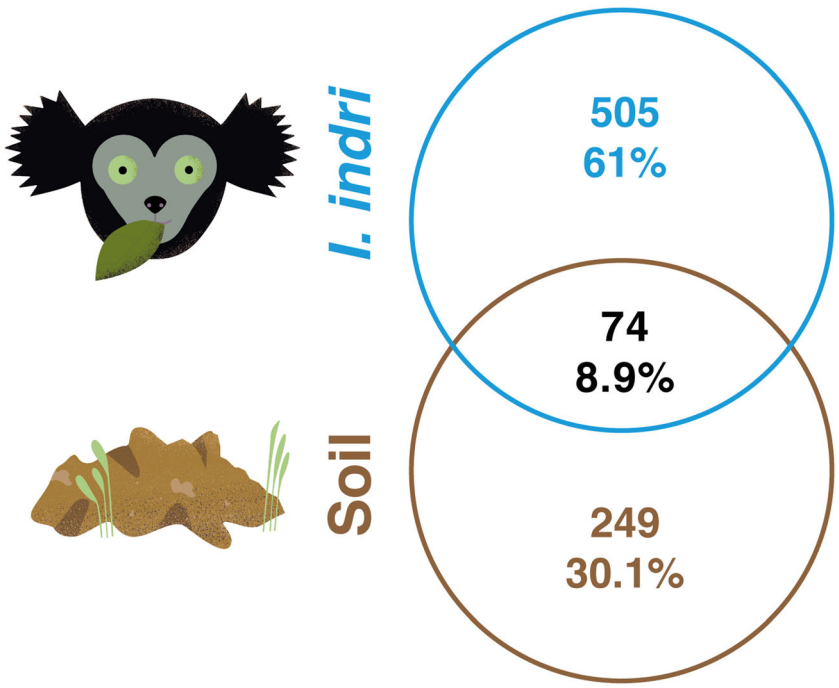

Fig. 2 Venn diagram showing the number and percentage of shared fungal OTUs between geophagic soils and indri. OTUs were defined by 97% sequence similarity

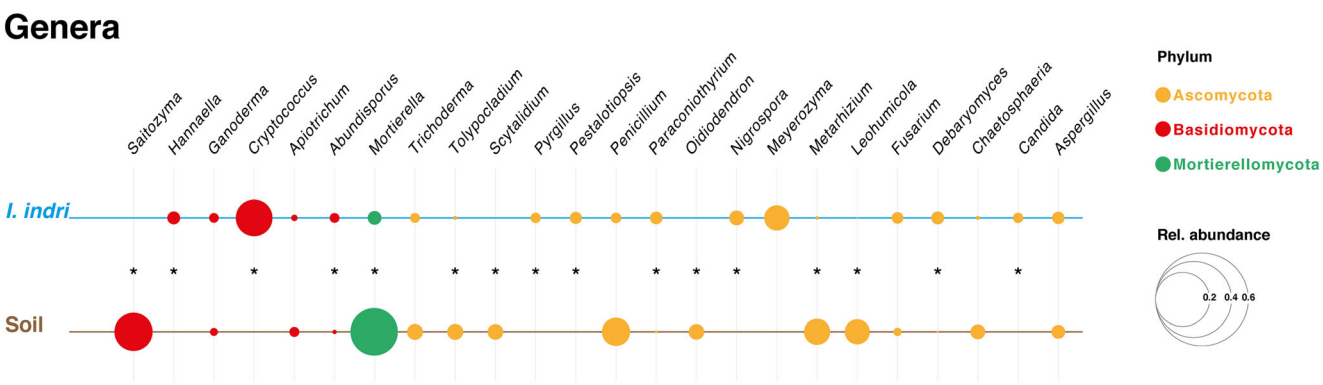

Fig. 3 Bubble plot representing the relative abundance of the most abundant Genera. The asterisk (*) indicates the significative difference between soil and indri samples (p value < 0.05 and LDA score > 2.0)

several secondary metabolites that play a crucial role in the initial destruction of complex organic compounds [58, 59]. Nevertheless, saprotrophic fungi could have a beneficial role in the production of enzymes necessary for the neutralisation of toxic compounds derived from the diet [60]. Although some environmental fungal species can be passengers or transient inhabitants of the indris' gut, they most likely affect the gut microbiome directly or indirectly (i.e. interaction with other microbes) [8, 61]. During geophagy, indris assumes soil microorganisms, which probably can colonise the intestine, at least in part and transiently. Thus, they effectively could fulfil a specific temporary or stable physiological role (e.g. plant polysaccharides, detoxification and production of bioactive or antimicrobial compounds) [39, 57]. Therefore, we cannot exclude that the continuous intake of soil microorganisms through geophagy could constitute for indris a sort of 'treatment' that they seek, relevant for their health.

Conclusion

Non-human primates are of particular interest for deepening our knowledge about bacterial microbiome research, but mycobiota of wild populations have been poorly explored. Recent findings have demonstrated the link between diet, habitat integrity and bacterial and fungal diversity in the host gut, rethinking the role of gut microbiota research as a tool for conservation [12, 62, 63]. As the microbial diversity may directly impact host health [64], the fungal diversity and the characteristics of the geophagic soil could play a crucial role in the indri's health. Thus, the soil may be considered a source of some fungal species and essential nutrients [8]. With this regard, protecting the lemur habitat integrity may be reflected in protecting the integrity of gut microbial diversity, especially in specialist primates, like the leaf-eating indris. Our findings expand the current knowledge of the gut fungal diversity and geophagy in wild non-human primates that could be a baseline

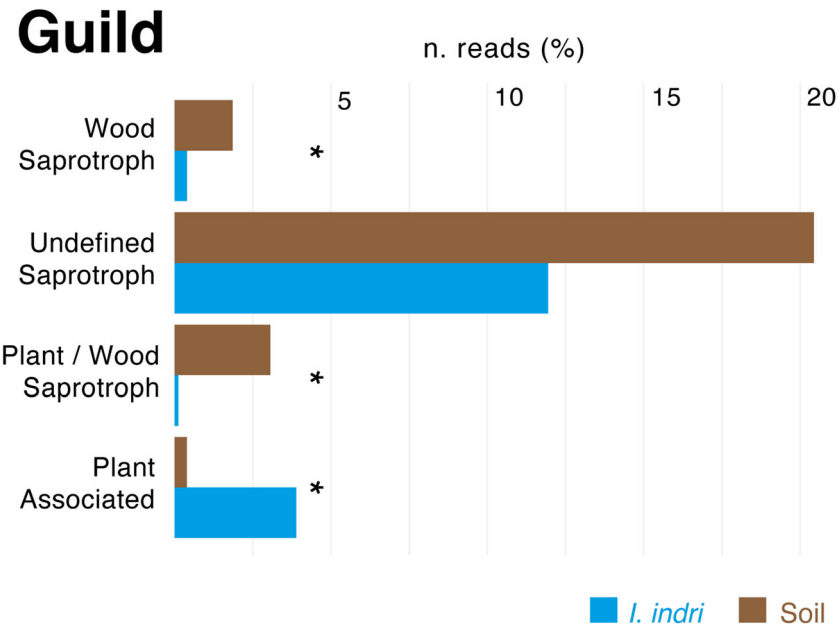

Fig. 4 Bar plots representing the relative abundance of predicted fungal functions/guilds. The asterisks (*), indicate the significative difference between soil and indri samples (p value < 0.05 and LDA score > 2.0) LDA score and p value of the most abundant genera are shown in Supplemental information (Tab. S1 and S2)

for further studies regarding the lemurs, including indris, conservation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-020-01677-5.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval The non-invasive methods used for faecal collections of wild indris adhere to the International Primatological Society (IPS) 'Principles for the Ethical Treatment of Non-Human Primates'. Field data collection protocols were reviewed and approved by Madagascar's Ministère de l'Environnement, de l'Ecologie et des Forêts (Permit 2018: N° 91/18/MEEF/SG/DGF/DSAP/SCB.Re). Field data collection protocols were also approved by GERP (Groupe d'Étude et de Recherche sur les Primates de Madagascar), the association governing research in the Maromizaha New Protected Area.

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Supplemental tables and figure

I like the way you eat it: Lemur (*Indri indri*) gut mycobiome and geophagy

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Contents

Fig. S1: Rarefaction curves of the indris A) and soil B) samples

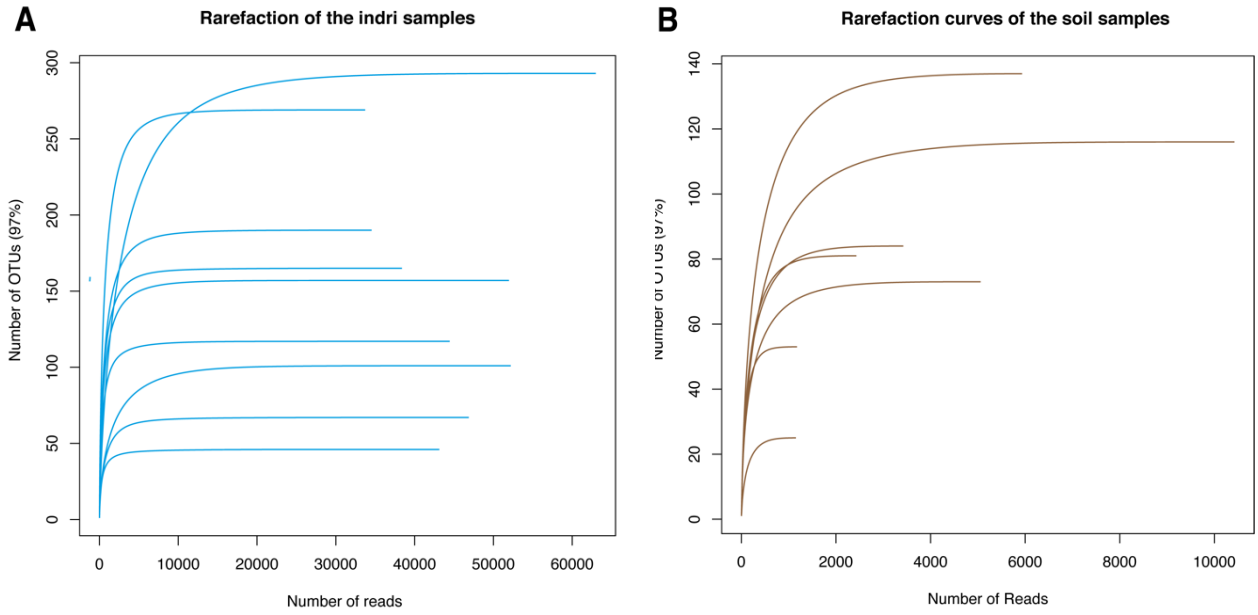
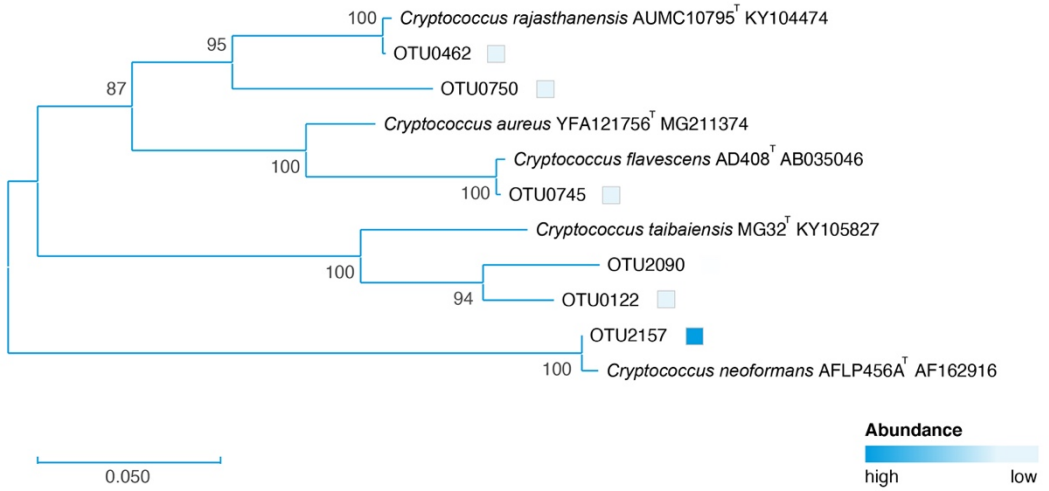


Fig. S2: Neighbour-joining phylogenetic tree of the *Cryptococcus* group and related OTU found in the indris' samples. Bootstrap percentages from 1000 replications are shown on the branches. Type strain (T) and GenBank accession numbers are indicated after the species name. *C. rajasthanensis*, *C. aureus*, *C. flavescens* and *C. taibaiensis* are newly described as *Papiliotrema rajasthanensis*, *P. aurea*, *P. flavescens* and *Vishniacozyma taibaiensis* respectively [1].



Tab. S1: LEfSe analysis identification of the most abundant fungal Genera.

Genus		LDA score	*p* value
Mortierella	Soil	3.8	> 0.01
Saitozyma	Soil	3.7	> 0.001
Cryptococcus	Indri	3.7	> 0.01
Leohumicola	Soil	3.4	> 0.001
Metarhizium	Soil	3.3	> 0.001
Scytalidium	Soil	3.2	> 0.001
Oidiodendron	Soil	3.2	> 0.01
Tolypocladium	Soil	3.1	> 0.01
Candida	Indri	3.0	> 0.01
Nigrospora	Indri	3.0	> 0.01
Pestalotiopsis	Indri	3.0	> 0.01
Paraconiothyrium	Indri	2.9	> 0.01
Abundisporus	Indri	2.9	> 0.05
Hannaella	Indri	2.9	> 0.05
Debaryomyces	Indri	2.9	> 0.01
Pyrgillus	Indri	2.9	> 0.01

Tab. S2: LEfSe analysis identification of the fungal guild. (Abbreviations: n.s., not significant).

Functional guild	Class	LDA score	*p* value
Plant associated	Indri	5.3	0.003
Undefined Saprotroph	/	/	n.s.
Plant Saprotroph-Wood Saprotroph	Soil	5.3	0.001
Wood Saprotroph	Soil	5.1	0.005

Reference

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5 General Discussion and Conclusions

5.1 Summary of major findings

Understanding the drivers of the gut microbiota of the animals and their relationship to the environment could be essential for planning strategies to conserve, monitor, and promote their health. Whether the gut microbiome facilitates the use of hard-to-digest food items, part of the diet of herbivores, it would be crucial to characterize the bacterial gut microbiome's shaping factors. The aims of this PhD project were to develop interdisciplinary studies in which conservation scientist and microbiome researchers work together to identify reliable microbial indicators that reflect the specific conservation needs of the host. The Seychelles giant tortoises (*Aldabrachelys gigantea*) and the "giant" lemur indris (*Indri indri*) were involved in this project. For the Seychelles giant tortoises, the study has involved tortoises in the wild but also in controlled environment at the Seychelles and in Italy. Regarding the study on indris, this has been focused on indris in the wild as the knowledge of the species is not enough to be able to manage it under human care. However, a step forward has been done. Assuming the link with the environment is stronger for this species than others, and as geophagy behavior has been observed on daily bases, a deeply investigation has been conducted also involving the environment. A characterization of the gut microbiota and mycobiota of the indris, but also of the geophagy soil, has been done.

The aim of the ***first study*** with the aim to characterize the gut microbiota of the Aldabra giant tortoise and to compare the microbiota of tortoises living under natural conditions, on the Seychelles Islands, with individuals living in controlled environments, in zoological and botanical gardens both in Italy and on the Seychelles Islands. Differences were reported regarding the bacterial gut community structure between tortoises in natural and in controlled environments. This study provided novel insights into the effects of different environmental conditions on the gut microbial communities of giant tortoises. These results could help to improve the management of giant tortoises under human care, thus enhancing ex-situ conservation efforts far from the species geographic range.

The aim of the ***second study*** was to analyze the gut microbiome composition of wild indris belonging to five different familiar groups in Madagascar to discover the potential drivers affecting host-microbial diversity. In addition, as Indris have never

been successfully bred under human care, suggesting that some behavioral and/or ecological factors are still not considered from the *ex-situ* conservation protocols, the chemical composition of geophagy and non-geophagy soil was analyzed to unravel the possible adaptive ecological value. The results provide a baseline for outlining some possible drivers, linked to the environment, responsible for the gut microbiome diversity in indris, thus laying the foundations for developing further strategies involved in indris' conservation.

The aim of the ***third study*** was to get a deeper knowledge of indri by investigating the linkages between geophagy soil and indris' gut mycobiome. The putative geophagy soil contribution to the fungal communities inhabiting the indris' gut was evaluated. An intimate connection between gut mycobiome and soil has been highlighted and the soil may be considered a source of some fungal species and essential nutrients.

5.2. Significance of results

Assuming that host-associated microbiota might help to develop a rapid ecological adaptation in response to changes in local environmental conditions. On the contrary, there are numerous factors that could determine the gut microbiome variation and some of them could be naturally occurred such as environmental changes. A limited number of studies have addressed the implications of changes in the microbiome for animal conservation. Possibly many factors relevant in conservation biology could affect the microbiome of animals including inbreeding, habitat fragmentation, change in climate, and effect of keeping animals in controlled environment. With the next generation sequencing and functional analysis of microbiomes it has become possible to test direct hypothesis on the importance of the microbiome in conservation biology (Bahrndorff et al., 2016). Studies have started to highlight the importance of the host-microbiome for conservation efforts (Amato et al., 2013; Bahrndorff et al., 2016; Stumpf et al., 2016; Hauffe and Barelli, 2019; Trevelline et al., 2019; West et al., 2019). These studies looked at host-microbiomes of endangered species and comparing groups that are affected or not by anthropogenic disturbances (e.g., comparing animals from the wild with individuals held in controlled environment, groups exposed to habitat fragmentation, or dietary shifts) (Menke et al., 2017; Jia et al., 2018; Han et al., 2019; van Leeuwen et al., 2020). The studies of my PhD project add to the recent panorama new data of the two herbivore giants, both living on islands

but belong to two different taxa, a tortoise and a lemur. Having investigate both scenarios, comparing Seychelles giant tortoises from the wild with individuals held in controlled environment, and groups of indris exposed to habitat fragmentation, it seems that microbiomes might play a larger role in future conservation biology, where the conservation of endangered species will be more dependent on populations held in zoological and botanical garden or aquaria.

5.3 Conclusion and implication for further research

The aim of present research is to highlight the relevance, possibilities and potential benefits of microbiome research for the field of conservation. *Ex-situ* conservation has been recognized to be crucial to guarantee the survival of the species but the effect of living in a controlled environment on the individuals should be minimized, according to the need of the species. Many threatened and endangered species undergo *ex-situ* breeding programmes to facilitate species recovery. Experiences suggest that besides positive projects where the *ex-situ* programme and reintroduction of the endangered species has been necessary to prevent extinction and accelerate recovery by helping to increase the number of individuals in the wild, there are unsatisfactory reintroduction projects which are not yet self-sustaining, where one remnant reintroduced population is likely to be extinct within the following few years. Research regarding the microbiota of the animals (bacteria, fungi, viruses and archaea), are fundamental as this microcosmos is linked to the entire organism health and survival. Despite the challenges associated with integrating microbiome research into current wildlife management practices, this field of research has much to offer to the conservation. Indeed, as the wild environment is affected by human activities, the microbiome research can be very useful also for *in-situ* conservation to detect the effects of humans on the environment and thus in wildlife. Conservation scientists often have great familiarity with the problems, challenges and opportunities that exist within their systems (e.g. the effect of land use practices on the gut microbiome of wild animals). In return, microbiome scientists can provide technical expertise while relaying the limitations of microbiome science. This study also highlighted the relevance of the habitat disturbance caused by humans on the animal species that shows soil consumption in their life. Different hypotheses are suggested to explain geophagy and all of them are important for the survival of the species. In a critically endangered

species, such as *Indri indri*, with its survival strictly linked to the soil consumption, both *ex-situ* conservation actions and micro and mycobiota research are fundamental for the conservation of the species.
If implemented properly, the ideas and techniques from both these fields can produce novel and meaningful results with the potential to increase our scientific understanding while advancing the field of wildlife conservation.

I am really convinced that this process might be useful to reach the goals of conservation, and this project can serve as a basis for further research.

This research represents an important step after an incredible three years of work done through the collaboration between Parco Natura Viva - Garda Zoological Park and University of Bologna University (DISTAL Department) and the Ministry of Environment, Energy and Climate Change, now Ministry of Agriculture, Environment and Climate Change and in particular the Seychelles National Parks Authority.
Moreover, the second part of my PhD project has been characterized by the collaboration with University of Turin (Department of Life Sciences and Systems Biology), Prof. Cristina Giacoma and her staff, Università Cattolica del Sacro Cuore in Rome and Faculty of Science and Technology, Free University of Bolzano. The collaboration between University of Turin and Parco Natura Viva started more than 20 years ago when the conservation project in Madagascar started. However, thank to my PhD project this collaboration had a new focus on a new tool for conservation: the microbiome. Having built the relationship based on the trust with local institutions of Madagascar over this long period has been useful to run the study. We followed the Madagascar example to start to build the relationship on the Seychelles Islands. Further research can be run to go in deep to investigate the effect on the animal health and welfare of each variable of the environment in which an individual leaves. As done for indris, an investigation of the microbiota of the soil in comparison to the microbiota of the tortoises might be developed. Fungi and Archaea should be also involved in further research to describe the entire microbiome of both Seychelles giant tortoises and Indris. General information on the human disturbance should also have to be added to this field of research.

I really hope that this PhD project could have provided the basis for further research that should go deeply in finding how to increase the welfare of the Seychelles giant tortoises in the controlled environment and to preserve them in the wild and how to guarantee a long life the Indri saving them from extinction.

5.4 REFERENCES

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