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ANDRESSA DIAS LEÃO

A glimpse into the first-ever sequenced gut microbiome of a South American wild canid: bacterial composition and antibiotic resistance genes

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I stand
on the sacrifices
of a million women before me
thinking
what can I do
to make this mountain taller
so the women after me can see farther

Rupi Kaur

SUMMARY

1 INTRODUCTION

The next-generation sequencing (NGS) technologies have been contributing to the human and other animals' microbiome studies. [1–3]. Through these studies, it is observed that the composition of the microbiota plays a key role in animal physiology (i.e. immune system, digestion, and development) [4–7]. Regarding the microbiome analysis, the *16S rRNA* sequencing provides quick and reduced computational and financial costs with scientifically relevant results from Bacteria and Archaea communities [8]. The analysis of bacteria communities through large scale sequencing of the *16S rRNA* encoding gene allowed us to understand, in a deeper manner, the relationships of these communities in spatial and temporal resolution from a single individual to a complete ecosystem [9].

In recent years, microbiome studies have been growingly applied to the field of wildlife conservation, an area that aims to understand and reduce the human impacts on biodiversity [10,11]. Ecosystem and habitat destruction, and the consequent fragmentation of natural habitats, result in a mixing of a wide variety of species, increasing contact between animals, including humans, which can increase the frequency of infectious diseases [12]. The anthropogenic impact, more specifically, can alter the gut microbiome, cause dysbiosis, and play an important role in the emergence and spread of antibiotic-resistant bacteria [13]. Due to the importance of microorganisms in animal diseases, and the imminent decline of wild populations, the maintenance of healthy microbiomes must become an integral part of conservation biology [14,15].

Empirically, this issue can be envisaged when one looks at wild canids, globally and in regional ecosystems, being even possible to identify the indirect effects of anthropogenic actions in the environment using metagenomic analysis. Anthropic actions in North America have reduced drastically the population of Red wolves [16]. The species is facing serious risks of being extinct - which has prompted scientific attention to their microbiome both in the wild and in captivity [17,18]. On the other hand, South America has a diversity of wild canids (11 in total), although still lacks studies that manage to understand the human impact on these animals. In Brazil, the maned wolf (*Chrysocyon brachyurus)* is facing a decline in its population because of agriculture [19], although no research using NGS seems to have been conducted to analyze the effects of this activity on their gut microbiome.

Pampa is one of the six biomes present in Brazil. It covers 1.76% to 2.07% of the Brazilian territory and stretches through Paraguay, Argentina, and Uruguay [20]. In the south of Brazil, the Pampa covers 63% of the state of Rio Grande do Sul [21]. Due to the favorable conditions for the expansion of large-scale agriculture and cattle ranching in the region, the Pampa biome has been undergoing major changes in its natural characteristics [21]. This biome harbor more than 100 species of mammals, which have been increasingly affected by these distortions; among them is the species *Lycalopex gymnocercus*, commonly known as Pampas fox [21]. The Pampas fox is a medium-sized canid with a size that ranges from 58.5 to 64 cm (in length) [22]. It has thick fur, a bulky tail, and a gray to yellowish color on the back; the belly and the inner surface of the limbs are pale gray to white [22]. *Lycalopex gymnocercus* can be found in the south of Brazil, Uruguay, Argentina,

Chile, and Bolivia (Figure 1) [23]. Regarding its eating habits, the Pampas fox is an omnivorous canid, and its generalist diet consists of fruits, insects, carcasses, and small mammals [24].

Figure 1: *Lycalopex gymnocercus* (on the left) and the species distribution (on the right). Source: Luciano Queiroz and International Union for Conservation of Nature (IUCN).

Generalist species are frequently more resistant to environmental changes and can serve as reservoirs of pathogens and vectors of zoonotic diseases [25]. As such, they can carry emerging resistant bacteria and genes, as well as facilitate their dissemination in the environment [26]. Because they are more flexible concerning diet, they may exhibit alterations in gut bacterial diversity from consuming anthropogenic food and, consequently, exhibit poorer health conditions [27]. Although the species *L. gymnocercus* has been classified as Least Concern (LC) by IUCN, modifications in the Pampa biome may alter its behavior, such as foraging habits, thus influencing the diet of the Pampas fox [23,28,29]. Also, the Pampas fox has been considered a vital livestock predator and has been actively persecuted by ranchers [22].

Due to the proximity of Pampas foxes to urban environments, it is important to detect the presence of antibiotic resistance genes (ARG) as a way of correlating the impact of anthropogenic interactions in food habits and bacterial composition [26,30]. In that way, we can evaluate the role of microorganisms in the health and conservation of wild canids [12,15]. Studying the microbiota of wild animals has become necessary, taking into consideration the prevention of future zoonoses, and also the protection of wildlife, the conservation of the environment, and consequently, the improvement of human health - as per the strategic objectives of the World Health Organization and its AMR Global Action Plan [31].

One way of carrying out monitoring is through the study of the animal microbiome. However, there is a lack of data regarding this species [22]; to our best knowledge, this is the first work analyzing the microbiome of a wild canid from South America. In this sense, we used high-throughput sequencing of *16S rRNA* to characterize the bacterial composition of four pampas foxes captured in the south of Brazil. We aimed to give a first-ever description of the Pampas foxes' gut microbiome and to analyze the level of anthropogenic contact by qualifying the presence of antibiotic resistance genes.

2 OBJECTIVES

2.1. Primary objective

To characterize the gut microbiome of *Lycalopex gymnocercus* and to identify the presence of antibiotic resistance genes

2.2. Secondary objectives

To identify the bacterial communities, in the phylum and family level, in the Pampas fox rectal samples.

To compare the gut microbiome diversity of the wild Pampas fox with the microbiome of other wild canids described in the literature.

To analyze the presence of the antibiotic resistance genes *msr*(C), *tet*(W), *bla*CTX-M, *bla*-TEM, *tet*(M), and *erm*(B) in the Pampas fox rectal samples.

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3 RESEARCH PAPER

The methods and results are discussed below as a scientific article, which will be submitted to the *Animal Microbiome Journal* (Impact Factor = 9.133). The figures and tables can be found at the end of the paper.

A glimpse into the first-ever sequenced gut microbiome of a South American wild canid: bacterial composition and antibiotic resistance genes

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ABSTRACT

The Pampa biome, located in the southern cone of South America, has been undergoing major changes due to the expansion of agriculture in the region. The Pampas fox (*Lycalopex gymnocercus*), a generalist-omnivorous canid, is one of the mammals that inhabits the Pampa biome. Generalist animals are generally more resistant to environmental changes and can serve as reservoirs of pathogens and vectors of zoonotic diseases. Although the species *L. gymnocercus* has been classified as Least Concern (LC) by the IUCN, modifications in the Pampa biome may alter its behavior, such as foraging habits, thus influencing the diet of the Pampas fox. Because they are more flexible concerning diet, they may exhibit alterations in gut bacterial diversity. In this study, we used high-throughput sequencing of *16S rRNA* to characterize the bacterial composition of four pampas foxes and analyzed the presence of six antibiotic resistance genes (ARGs), aiming to give a first look into the Pampas foxes' gut microbiome and analyze the level of anthropogenic contact. Regarding the bacterial composition, the dominant phylum observed was Proteobacteria. All samples were negative for the presence of the ARGs *msr*(C), *bla*CTX-M, and *bla*-TEM. Four samples presented the gene *tet*(M). The high abundance of Proteobacteria and the presence of *tet*(M) could be related to anthropic actions. Our study reinforces the importance of conducting research related to the impact of human activities on the Brazilian Pampa biome.

Keywords: Pampas fox; *Lycalopex gymnocercus*; wild canids; gut microbiota; antibiotic resistance; conservation biology.

INTRODUCTION

During the past years, microbiome studies have been growingly applied to the field of wildlife conservation [1,2]. Ecosystem and habitat destruction, and the consequent fragmentation of natural habitats, result in a mixing of a wide variety of species, increasing contact between animals, including humans, which can boost the frequency of infectious diseases [3]. Due to the importance of microorganisms in animal diseases, and the imminent decline of wild populations, the maintenance of healthy microbiomes must become an integral part of conservation biology [4,5].

In Brazil, the Pampa is one of the six biomes and comprehends 1.76% to 2.07% of its territory [6]. In the south of Brazil, the Pampa covers 63% of the state of Rio Grande do Sul [7]. Due to the expansion of large-scale agriculture and cattle ranching in the region, mainly due to the favorable conditions for their implementation, the Pampa biome has been undergoing major changes in its natural characteristics [7]. This biome harbor more than 100 species of mammals, which have been increasingly affected by these distortions; among them is the species *Lycalopex gymnocercus*, commonly known as Pampas fox [7]. The Pampas fox can be found in the south of Brazil, Uruguay, Argentina, Chile, and Bolivia [8]. Regarding its eating habits, the Pampas fox is an omnivorous canid, and its generalist diet consists of fruits, insects, carcasses, and small mammals [9].

Generalist species are frequently more resistant to environmental changes and can serve as reservoirs of pathogens and vectors of zoonotic diseases [10]. As

such, they can carry emerging resistant bacteria and genes, as well as facilitate their dissemination in the environment [11]. Because they are more flexible concerning diet, they may exhibit alterations in gut bacterial diversity from consuming anthropogenic food and, consequently, exhibit poorer health conditions [12]. Although the species *L. gymnocercus* has been classified as Least Concern (LC) by IUCN, modifications in the Pampa biome may alter its behavior, such as foraging habits, thus influencing the diet of the Pampas fox [8, 13]. Also, the Pampas fox has been considered a vital livestock predator and has been actively persecuted by ranchers [14].

Due to the proximity of Pampas foxes to urban environments, it is important to detect the presence of antibiotic resistance genes (ARG) as a way of correlating the impact of anthropogenic interactions in food habits and bacterial composition [11, 15]. In that way, we can evaluate the role of microorganisms in the health and conservation of wild canids [3, 5]. Studying the microbiota of wild animals has become necessary, taking into consideration the prevention of future zoonoses, and also the protection of wildlife, the conservation of the environment, and consequently, the improvement of human health - as per the strategic objectives of the World Health Organization and its AMR Global Action Plan [16].

One way of carrying out monitoring is through the study of the animal microbiome. However, there is a lack of data regarding this species [13]. To our best knowledge, this is the first work analyzing the microbiome of a wild canid from South America. In this study, we used high-throughput sequencing of *16S rRNA* to characterize the bacterial composition of four pampas foxes captured in the south of Brazil. We aimed to give a first-ever description of the Pampas foxes' gut

microbiome and to analyze the level of anthropogenic contact by qualifying the presence of antibiotic resistance genes.

MATERIAL AND METHODS

Study area and Samples collection

Pampas foxes were captured in four different sites near the city of Candiota, Rio Grande do Sul State, Brazil (31°33'06.73"S; 53°40'40.63"W), as shown in Figure 1. Rectal swabs were collected from wild Pampas foxes (n = 4) by veterinarians after being captured with the assistance of Tomahawk traps and anesthetized via intramuscular (100 mg/mL of ketamine hydrochloride and 20 mg/mL of xylazine hydrochloride). All animals were clinically healthy (e.g. rectal temperature, heart rate, and respiratory rate) and were classified according to gender, age, and weight. The summary of the sample's information is shown in Table 1.

These procedures were made with the authorization of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio). The protocol was approved by the Information Authorization System in Biodiversity (SISBIO) number 0200 1.007 9 10 12006-32. After the collection of samples, the animals were returned to their habitats in healthy conditions. Rectal swabs were stored in Stuart transport medium (Kasvi, Paraná, Brazil), and transported to our laboratory, where they were kept at 4°C until DNA extraction.

DNA extraction

Rectal swabs samples were suspended in 2mL of saline solution 0,85% and kept under agitation (100 rpm) for 2 hours (37°C ±1ºC). We used 1,5mL of the solution for DNA extraction. According to the manufacturer's instructions, we extracted the total DNA of each sample using MoBio's PowerSoil DNA extraction kit (ThermoFisher Scientific). DNA concentration was determined using the Qubit, and its quality was verified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR-amplification of bacterial 16S rRNA gene and sequencing

For the characterization of the bacterial community of each sample, we used the primers 515F and 806R [17] to amplify the V4 region of the *16S rRNA*. The samples were further sequenced using 316 chips - PGMTM Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's specifications.

Samples were PCR-amplified with barcoded primers linked with the Ion adapter "A" sequence and Ion adapter "P1" sequence to obtain a sequence composed of adapters plus primers. We performed PCR assays with the Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA), in a volume of 25 µL containing 1 × High Fidelity PCR buffer, 2U of Taq Polymerase, 2 mM $MgSO_4$, 0.2 mM dNTP Mix, 25 µg of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA), 0.1 µM of each primer and approximately 50 ng of DNA template and ultrapure water to complete the volume.

The PCR condition of the first cycle was 94ºC (5 min), while the subsequent 30 cycles were: 94ºC (45 s), 56ºC (45 s), and 68ºC (1 min), with a final extension of

68ºC (10 min). Afterwards, the sequencing was performed at the Federal University of Pampa (UNIPAMPA, São Gabriel, RS, Brazil). We purified the amplicons using Agencount AMPure Beads (Beckman Coulter), and the library preparation was carried out with the Ion OneTouchTM 2 System fitted with the Ion PGMTM OT2 400 Kit Template (Thermo Fisher Scientific, Waltham, MA, USA) using an initial amount of 100ng of PCR product. Since we have sequenced all samples in a multiplexed PGMTM run, barcode sequences were applied for the identification of each sample from the output.

Bacterial community and bioinformatics analyses

We conducted all analyses using the galaxy@pasteur platform [18]. We evaluated the raw data quality with FastQC [19] and constructed a report with MultiQC [20]. Elimination of the adapters was done with Cutadapt v.2.3 [21], and the quality-filtered sequences were imported into the FROGS (Find Rapidly OTUs with Galaxy Solution) pipeline [22] to obtain the Operational Taxonomic Units (OTUs). The sequences were filtered by length (250–300 bp) and then pooled into OTUs with SWARM [23] with the distance parameter d =3.

Chimeras were removed with VSEARCH [24] and OTUs corresponding to at least 0.1% of the whole dataset were maintained. These steps resulted in the retention of OTUs, which were affiliated with SILVA 132 SSU databases [25], delimited at 97% identity [26].

We perform the statistical analyses with the FROGSSTAT, which utilizes R v.4.0.3 and the phyloseq package (v1.28.0) [27]. For the alpha diversity analysis, we used the *'Phyloseq Alpha diversity'* and selected the following indexes: Chao1, Shannon, Simpson, and Inverse Simpson. The relative abundance of

species present in the samples was plotted with the *'Phyloseq Composition Visualization'* function; a phylogenetic tree was also created utilizing the same function. To analyze the beta diversity, we used the *'Phyloseq Beta Diversity'* function to construct a distance matrix (Jaccard index), and with the *'Phyloseq structure visualization'* we built an ordination plot (MDS/PCoA) and a heatmap of the OTUs. Statistics were performed with ANOVA.

Antibiotic resistance genes analysis

We used the total DNA to analyze the presence of antibiotic resistance genes commonly in clinical and environmental samples. All the information regarding the genes, primer sequences, pair of bases (pb), and references, can be found in Supplementary Table 1. The ARG evaluated were: *erm*(B), *msr*(C), *tet*(M), *tet(*W), *bla* CTX-M, and *bla*TEM.

PCR amplifications were conducted with a total volume of 25 µL containing: 100 ng of template DNA, 1 X reaction buffer (Ludwig Biotechnology), 0.4µM of each primer (Ludwig Biotechnology), 1.5mM MgCl2, 200µM of dNTPs (Ludwig Biotechnology), 1U Taq DNA polymerase (Ludwig Biotechnology), and MilliQ water. We performed the PCRs in a conventional thermocycler (Applied Biosystems 2720 Thermal Cycler) according to the following program: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, appropriate annealing temperature for each primer for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5min. We analyzed the DNA fragments amplified in 1.5% (w/v) agarose gels stained with SYBR® Safe DNA Gel and visualized on a photo-documenter.

RESULTS

We obtained a total of 125,621 high-quality reads. The rarefaction plot is available in Figure 2a. We apply a filter to obtain the significant OTUs only (0.1%). After the filter application, 99.4% OTUs were removed and 291 OTUs remained. OTUs resulted in 11 different bacterial phyla in total (Figure S1). Through a phylogenetic tree, it is possible to visualize how the OTUs were assembled (Figure S2). Some of the samples presented multi-affiliations, which means that the database could not classify precisely some OTUs. Also, some OTUs were classified as *unknown family*. Differences in bacterial composition were observed among samples, especially when comparing the female (LG2) to the male samples. The dominant phylum was *Proteobacteria* in the males (58.9–65.1%), while LG2 presented a notable higher abundance of *Fusobacteria* (70%) (Figure 3a). *Bacteroidetes* abundance was considerably low and similar among all four samples, varying between 1.56% and 1.24%. Contrariwise, *Firmicutes* showed differences among the Pampas foxes' gut. Only one of the male samples was *Firmicutes* enriched.

From the taxonomic family level, a total of 32 families were observed. However, most of the groups presented low relative abundances >1%. There was a significant number of multi-affiliations in the male samples (Figure 3b). From the families identified, *Enterobacteriaceae* and *Fusobacteriaceae* were present in all samples. Following the same pattern as with the phyla, LG2 presented *Fusobacteriaceae* as the main family (70%). The *Comamonadaceae* family was observed in sample LG4 (14.1%).

Alpha diversity metrics (i.e., Shannon, Chao1, Simpson, and InvSimpson indices) did not exhibit statistical significance ($p>0.05$) in the bacterial composition of the Pampas fox samples analyzed in our study (Figure 2b). The beta diversity distance matrix (binary Jaccard distance, Figure 4) was used in the construction of the Multidimensional scaling (MDS), as a way of visualizing the level of similarity between the samples. The most diverse samples regarding the bacterial composition were LG1 and LG3, although all of them were considerably divergent from each other (Figures 5 and 6). Looking at the heatmap, the occurrence and frequencies of OTUs were more similar between the male samples when compared to the female (Figure 6). Although the mean distances between males and female were calculated, no statistically significant differences were observed.

Regarding the ARG analysis, all samples were negative to the presence of *msr*(C), *bla*CTX-M, and *bla*-TEM. The four samples presented the gene *tet*(M). LG1 and LG3 were positive for *tet*(W). Only LG4 had a positive result for *erm*(B).

DISCUSSION

We sequenced the V4 rRNA region of four rectal swab samples from Pampas foxes, a South American wild canid, to analyze the gut bacterial composition. Our results differ from most studies with wild canids, showing *Proteobacteria* as the most abundant phylum in the gut microbiota of Pampas foxes (Figure 7) [12, 34–51]. Previous studies have shown that the gut microbiome of wild canids usually presents a higher abundance of *Bacteroidetes, Firmicutes,* and/or *Fusobacteria*, as shown in Figure 7. In consonance with these studies, other paper analyzing the microbiota of

domestic dogs also showed these three phyla as preeminent in the gut/fecal microbiota of healthy dogs [52].

Proteobacteria is the most diverse bacterial phylum and is commonly present in the gut microbiota of healthy mammals [53,54]. In our study, we found *Proteobacteria* as the dominant phylum in all the males analyzed. In humans, *Proteobacteria* are mainly associated with diseases [53]. The *Enterobacteriaceae* family, more specifically, has been linked with chronic enteropathies [55]. In animal health, they are frequently highlighted as a microbial group of particular concern as they include several clinically important gastrointestinal pathogens, such as *Escherichia coli, Campylobacter jejuni, Klebsiella pneumoniae, Salmonella typhimurium,* and *Yersenia enterocolitica* [56].

The composition and diversity of the gut microbiome are influenced by a wide range of biological processes, such as social interactions, the host's evolutionary history, and diet [57,58]. Pampas foxes are omnivorous, and their diet varies according to food availability and region [59]. Castillo *et al.* 2011 performed a study analyzing the diet of Pampas foxes in the Chaco region, Argentina [60]. The results from [60] showed that adults eat insects and fruits, bringing more nutrient food to their cubs (i.e. rodents). According to this study, one of the fruits that Pampas foxes eat is from the genus *Prunus* sp. Plum trees (*Prunus domestica*) are one of the native species of this genus that can be found in the Pampa biome [61]. A study analyzing the microbiome of *Prunus* sp., discovered that *Proteobacteria* presented an abundance of 94% [62]. Also, the microbiome of insects is mainly composed of *Proteobacteria* [63]. These findings corroborate

the hypothesis that the prevalence of this phylum can be a result of the Pampas fox diet.

Regarding LG4, a cub, its bacterial composition could also be related to the early microbiome composition observed in humans and mammals. Facultative anaerobes, including *Proteobacteria*, are among the earliest colonizers and dominant members in the neonatal gut [53]. The phylum plays a key role in preparing the gut for successive colonization by the strict anaerobes required for healthy gut function [53]. After birth, the dominant phyla in the feces of mammals, such as tiger cubs, were *Proteobacteria, Firmicutes,* and *Cyanobacteria* [64]. The abundance of *Proteobacteria* tended to decrease gradually throughout their early life [64].

We cannot ignore the fact that the predominance of *Proteobacteria* could also be a result of anthropogenic interference. Biles *et al.* (2021) performed a study analyzing the scat microbiome of red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) in two parks in Virginia, United States [38]. Both wild canids presented high abundances of *Proteobacteria* and low abundances of *Firmicutes*. They hypothesized that their findings could indicate stress and poor health conditions, especially in the coyotes that live in the more developed park, Manassas National Battlefield Park (MANA).

The Pampa biome, which represents a large proportion of the Pampas fox's distribution range, have been affected by extensive cattle breeding and agriculture [6]. Approximately 0.1% of the original 500,000km² range remains unaffected. Due to the species' adaptability, the Pampas fox seems able to withstand the loss and degradation of its natural habitat and hunting pressure [13]. Nevertheless, Caruso *et al.* (2016) showed that even species considered

more adaptable, such as the Pampas fox and the Molina's hog-nosed skunk have shown some type of negative association with areas with human presence in the Pampa biome [65]. In Candiota, more specifically, 73,234 hectares are destined for agriculture and livestock production, which corresponds to 78.4% of its total area [66].

The sample LG2 showed *Fusobacteria* as the frequent phylum. *Fusobacteria* seems to be related with inflammatory bowel disease (IBD) and colorectal cancer in humans but not necessarily in dogs [67]. Interestingly, *Fusobacteria* has been associated with healthy dogs [68]. Also, its high abundance can be related to the high consumption of meat [54]. Nelson *et al.* 2014 showed that *Fusobacteria* were present at high abundances in canines, when compared to other terrestrial mammals [69].

Although no statistically significant differences were observed, the divergence observed between samples may be explained by the habits of these canids. Pampas foxes tend to be solitary animals, being in pairs only between the mating season [13]. In that sense, the availability of different nutritional sources found during foraging may favor the supply of different bacterial groups. Also, individual variations in the microbiome profile exist and should be considered especially when extrapolating findings from small sample groups.

All samples analyzed in this study were positive for the gene *tet*(M), and two were positive for the gene *tet*(W). Tetracycline-resistant genes were also found in Pampas fox from Argentina [70]. In their research, in consonance with our study, they found tetracycline ARG as the most prevalent ARG group, with almost 85% of foxes being positive for at least one *tet* gene. Sample LG4 also had a positive

result of the *erm*(B) gene, which confers cross-resistance against macrolides, lincosamides, and streptogramin [71]. This gene has been described in a wide variety of bacteria both in humans and animal isolates [30, 72]. Interestingly, the *erm*(B) gene is often linked with the *tet*(M) gene [71].

The presence of tetracycline-resistant genes can be explained by the fact that this antibiotic has been widely used in medicine for treatment, but also as a growth promoter in livestock production [73]. Those genes (*tet*) have been found in a variety of bacteria present in human and livestock-impacted environments [74-76]. Another important fact to consider is that the Candiota region presents coal mining activities, due to its soil (rich in coal and limestone) [66]. The production of coal might facilitate the proliferation of ARGs due to the ionic liquid used in the process of coal liquidation [77]. Many heavy metals can also increase the proliferation of antibiotic resistance due to their antimicrobial properties [78].

The limitation of our study is the lower number of samples, due to the difficulty of obtaining samples from wildlife. We understand that the lower number of samples probably influenced the statistical power of our analysis. Notably, capturing and handling wild animals requires specialized equipment, the consideration of animal welfare concerns, and the efforts of experienced biologists and wildlife technicians to plan and study suitable capture methods. Considering it, the number of animals evaluated in the present study should be well-considered, although the results should be interpreted with caution. Our study reinforces the importance of conducting research related to the impact of human activities on the Brazilian Pampa biome.

CONCLUSION

We present an overview of the Pampas fox microbiome. This study was the pioneer in identifying the microbiome in canids from South America, mainly in Brazilian biomes. Therefore, the analysis of the microbiome and resistance genes gives us clues about the impact of anthropic action in wild species. Studies such as the one presented here bring insights to understanding the conservation of local fauna. Hopefully, our study will become a foundation for new studies concerning the welfare of wild animals in the Pampa biome.

Data availability

All codes are available at the galaxy@pasteur platform. Additional information can be found at https://github.com/pampasfox/Pampasfoxdata. Sequences have been submitted and published to the NCBI database under accession number PRJNA827860.

Conflicts of interest

The authors declare that they do not have any disclosures.

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TABLES AND FIGURES

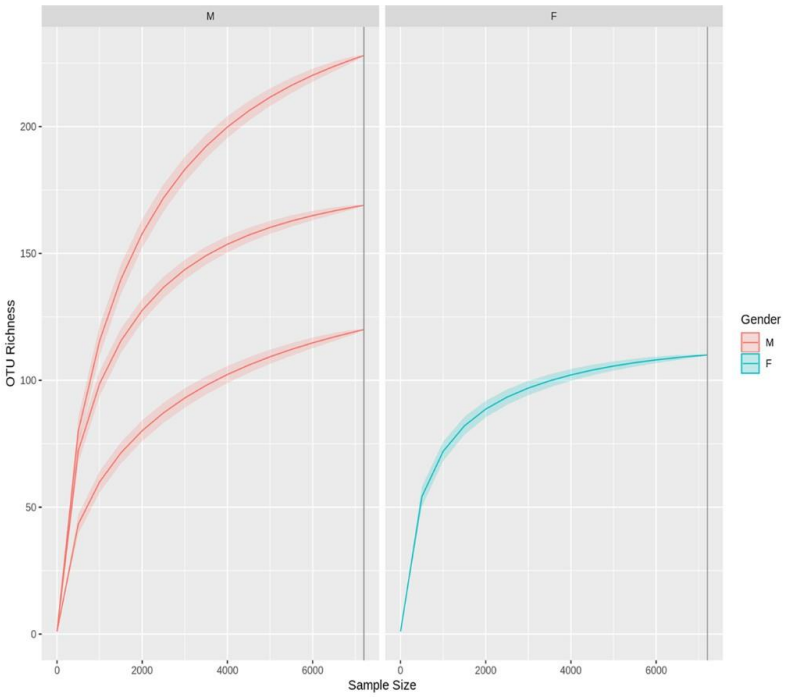
Table 1: Description of the Pampas fox samples evaluated in this study.

Species	Sample ID*	Sex	Age	Weight (kg)	Collection site	Collection date
Pampas fox (*L. gymnocercus*)	LG1	Male	Adult	5.22	Candiota City (Site 1)	10/12/2016
	LG2	Female	Young	3.95	Candiota City (Site 2)	15/12/2016
	LG3	Male	Adult	4.88	Candiota City (Site 3)	15/12/2016
	LG4	Male	Cub	1.45	Candiota City (Site 4)	13/12/2016

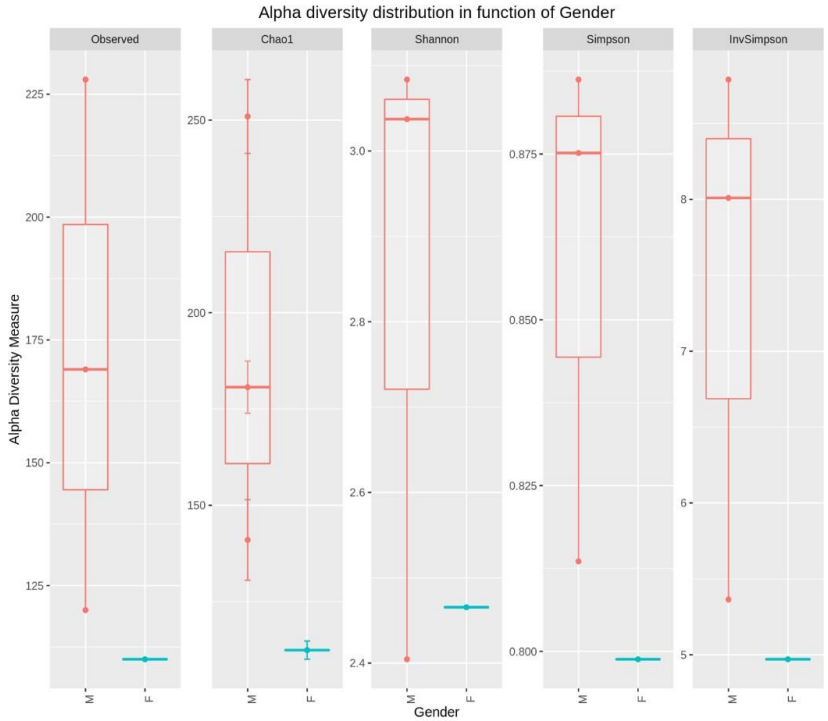
*LG1= *L. gymnocercus* sample 1; LG2= *L. gymnocercus* sample 2; LG3= *L. gymnocercus* sample 3; LG4= *L. gymnocercus* sample 4.

Figure 1: Samples collection site. Brazil is shown in the left highlighted in light grey. Rio Grande do Sul State is shown, detached from the main map, in the center. The collection sites in Candiota city are amplified. **Site 1, LG1:** Lat. 31°28'34.28"S, Long. 53°48'45.61"W. **Site 2, LG2:** Lat. 31°29'00.60"S, Long. 53°48'41.70"W. **Site 3, LG3:** La 31°28'18.52"S, Long. 53°49'8.32"W. **Site 4, LG4:** La 31°28'37.62"S, Long. 53°48'59.23"W.

Figure 2: Quantification of microbial communities. **2a:** rarefaction curve. **2b:** Alpha diversity barplot.



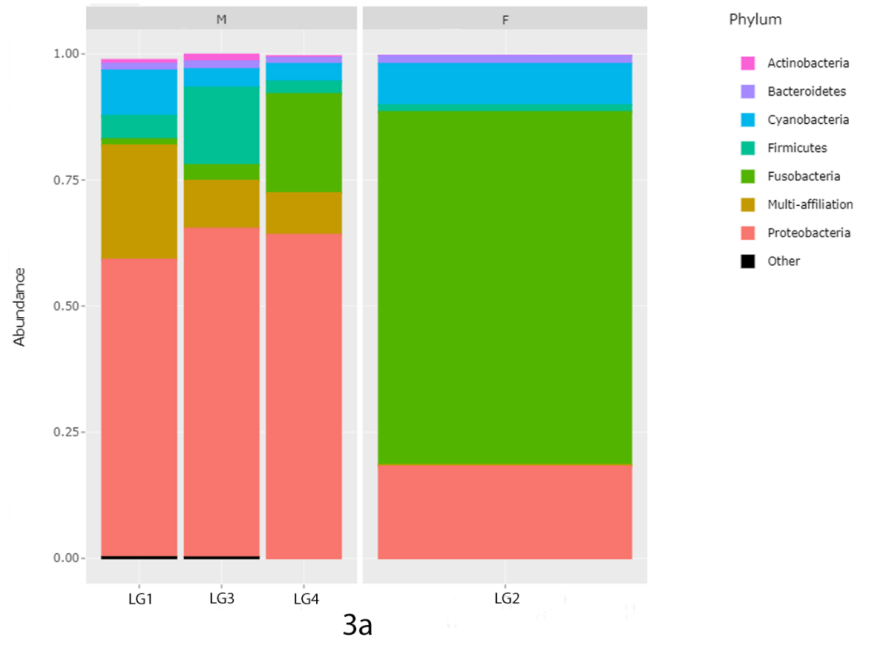
2a



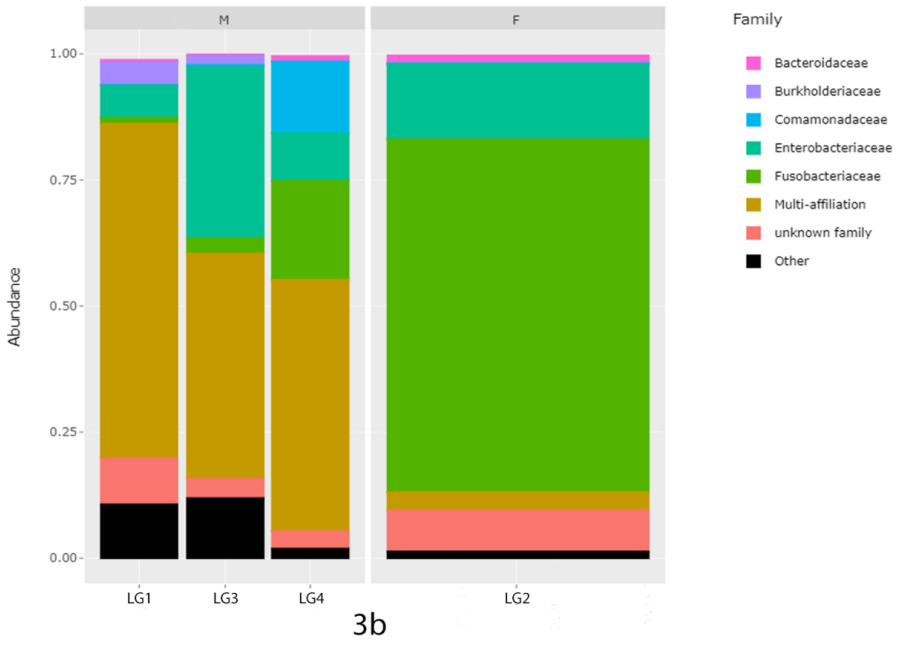
2b

M= Male. F= Female.

Figure 3: Barplots of the bacterial composition. **3a:** Phylum level. **3b:** Family level.



3a



3b

Table 3: Antibiotic resistance genes (ARG) present in Pampas fox (*Lycalopex gymnocercus*) microbiota.

ARG	Sample			
	LG1	LG2	LG3	LG4
erm(B)	-	-	-	+
msr(C)	-	-	-	-
tet(M)	+	+	+	+
tet(W)	+	-	+	-
*bla*CTX-M	-	-	-	-
bla-TEM	-	-	-	-

Figure 4: Jaccard plot of similarity index and distance between samples.

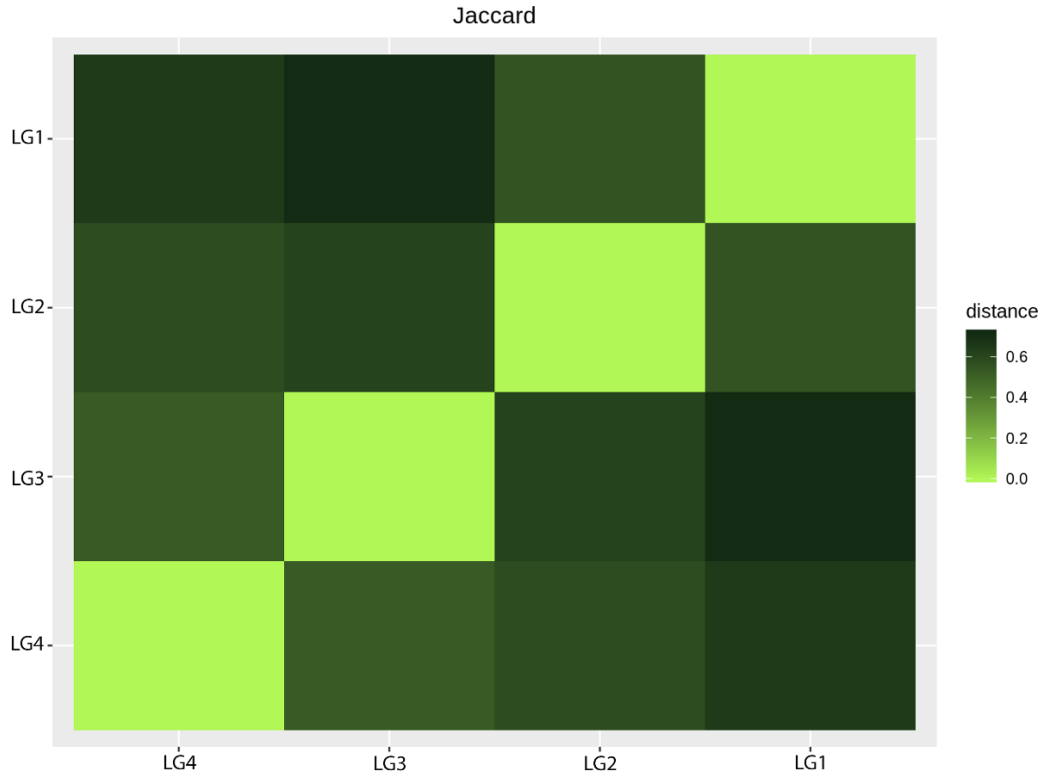
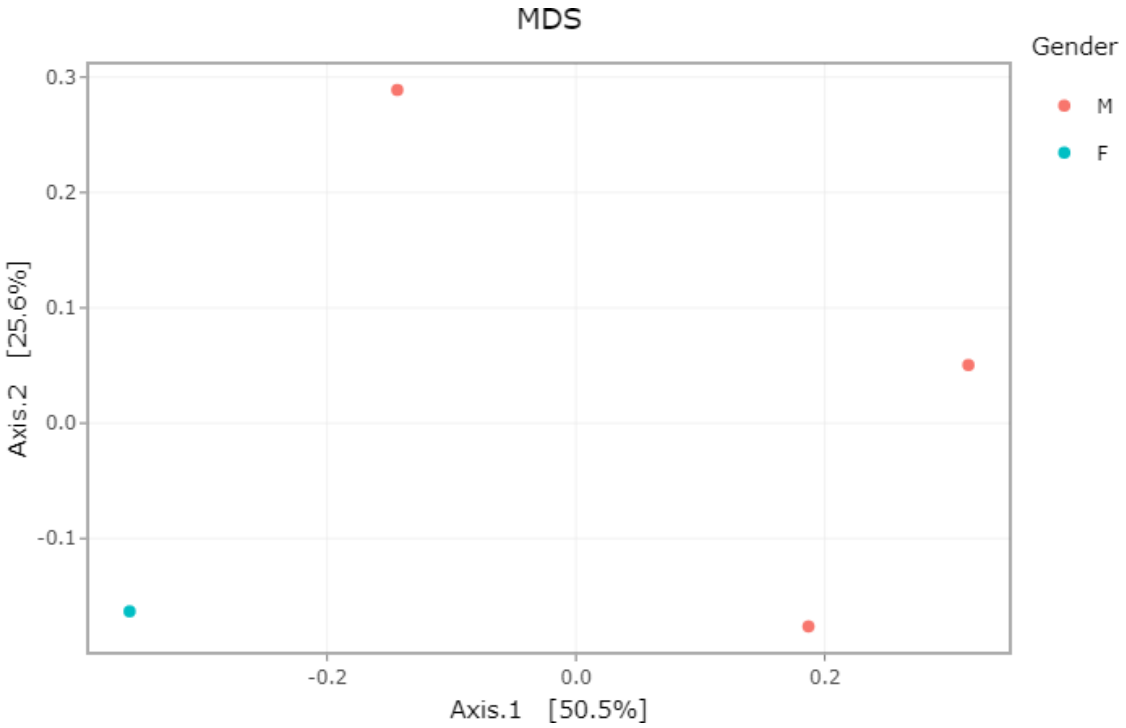


Figure 5: Principal Coordinates Analysis (PCoA – MDS) plot.



M= Male. F= Female.

Figure 6: Heatmap of the contribution and diversity of the taxa analyzed.

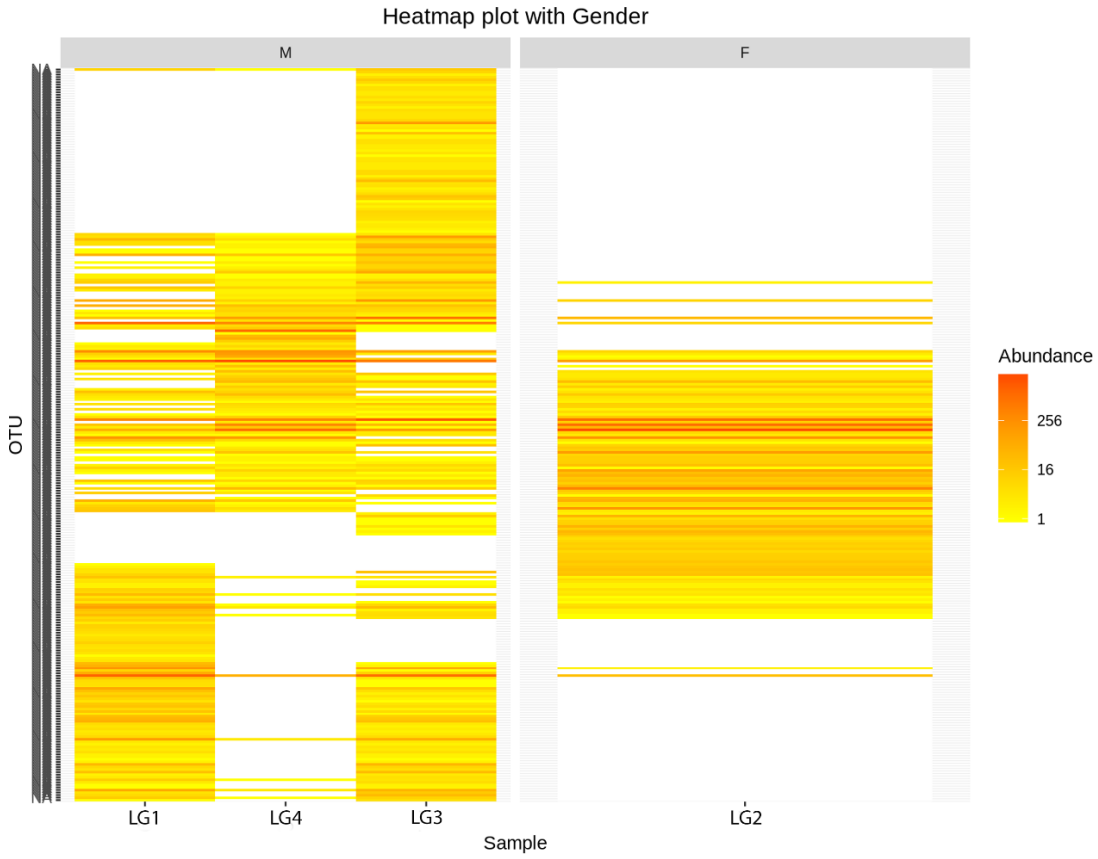
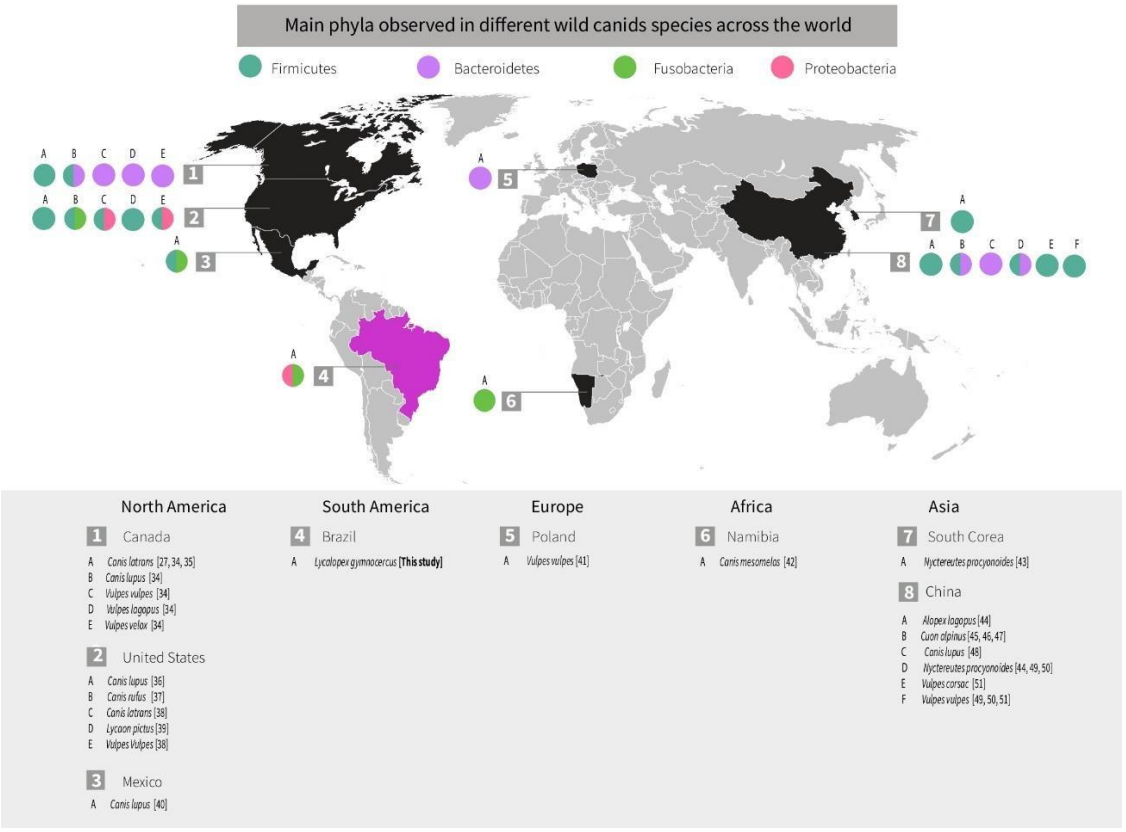


Figure 7: Overview of the main phyla observed in different studies with wild canids performed worldwide between 2016 and 2022. Phylum indicators expressed in colors related to qualitative taxonomic diversity. Elaborated by the author.

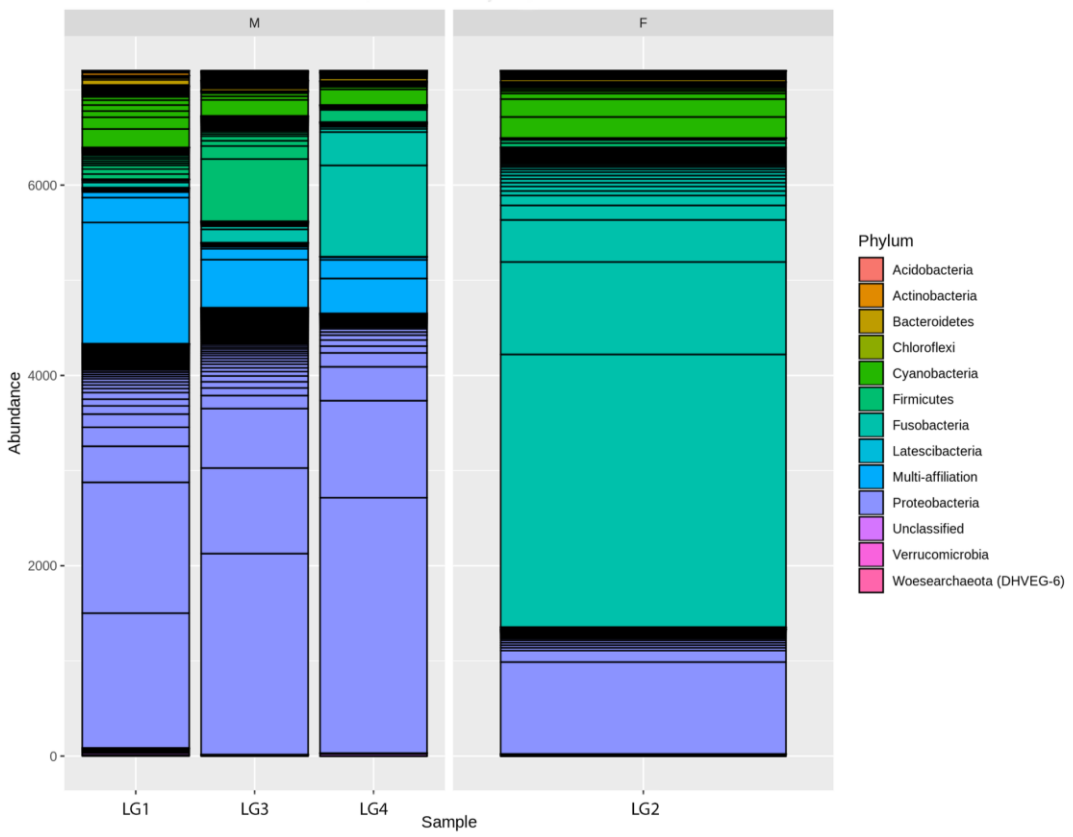


Supplementary Table 1: Conditions of the amplification of the ARGs used in this study.

Gene	Primers sequences (5' – 3') (F and R*)	Size (pb**)	Reference
***erm*(B)** (F)	GAAAAGGTACTCAACCAAATA	639pb	[28]
***erm*(B)** (R)	AGTAACGGTACTTAAATTGTTTAC		
***msr*(C)** (F)	AAGGAATCCTTCTCTCTCCG	342pb	[29]
***msr*(C)** (R)	GTAAACAAAATCGTTCCCG		
***tet*(M)** (F)	GTTAAATAGTGTTCTTGGAG	660pb	[30]
***tet*(M)** (R)	CTAAGATATGGCTCTAACAA		
***tet*(W)** (F)	GAGAGCCTGCTATATGCCAGC	167 pb	[31]
***tet*(W)** (R)	GGGCGTATCCACAATGTTAAC		
***bla*CTX-M** (F)	SCSATGTGCAGYACCAGTAA	585pb	[32]
***bla*CTX-M** (R)	ACCAGAAYVAGCGGBGC		
***bla*-TEM** (F)	GCACGAGTGGGTTACATCGA	310 pb	[33]
***bla*-TEM** (R)	GGTCCTCCGATCGTTGTCAG		

*F= Forward. R= Reverse.
**pb= pair of bases.

Supplementary Figure 1: Barplot of phyla composition.



Supplementary Figure 2: Phylogenetic tree of phyla composition.