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The potential effect of the oral bacterial community in Melanophryniscus admirabilis (admirable red-belly toads) conservation

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Abstract: *Melanophryniscus admirabilis* (admirable red-belly toad) is a microendemic and critically endangered species found exclusively along 700 meters of the Forqueta River, in a fragment of Atlantic Forest in southern Brazil. One of the greatest concerns regarding the conservation of this species is the extensive use of pesticides in areas near their natural habitat. In recent years, the adaptation and persistence of animal species in impacted environments have been associated with microbiota. Therefore, the current study aimed to characterize the oral bacterial community of wild *M. admirabilis* and to address the question of how this community might contribute to toad's adaptation in the anthropogenic environment and its general metabolic capabilities. In the present study, 11 oral samples collected from wild *M. admirabilis* were characterized and analyzed via high-throughput sequencing. A total of 181,350 sequences were obtained, resulting in 16 phyla, 34 classes, 39 orders, and 77 families. Proteobacteria dominated (53%) the oral microbiota of toads followed by Firmicutes (18%), Bacteroidetes (17%), and Actinobacteria (5%). No significant differences in microbial community profile from among the samples were reported, suggesting that the dietary restriction may directly influence the bacterial composition. Functional inference of microbiome was performed using PICRUSt2 and important pathways, such as xenobiotic degradation pathways to pesticides and aromatic phenolic compounds were detected, suggesting that the bacterial communities may have important roles in *M. admirabilis* health and survival in the anthropogenic environment. Overall, our results have important implications for the conservation and management of this microendemic and critically endangered species.

Keywords: High throughput sequencing; amphibian; bacteria; xenobiotic; anthropogenic action.

1. Introduction

Amphibians are highly sensitive to environmental changes, being the most threatened vertebrate group, with approximately 40% of the 8261 known species [1] listed in some threatened category [2,3]. The anuran genus *Melanophryniscus* (Bufonidae) is composed of 29 species of small

toads that are geographically restricted to South America, occurring in Brazil, Paraguay, Bolivia, Uruguay, and Argentina [4]. Many species have restricted distributions and are globally threatened, near threatened [5], or data deficient [3]. Among the 22 species of *Melanophryniscus* found in Brazil, *Melanophryniscus admirabilis* (admirable red-belly toad) is a microendemic species found exclusively along 700 meters of the Forqueta River in a fragment of Atlantic Forest in southern Brazil [6]. *M. admirabilis* is one of the largest species in the genus (females grow up to 40 mm while males are smaller) [4]. They are easily distinguishable by their green dorsum with a black belly as well as contrasting yellowish glands and red palms, soles, and inguinal region [6]. Its conspicuous coloration, associated with unken reflex behavior is supposedly a warning signal for potential predators, indicating that they are toxic due to their skin`s alkaloid compounds. The alkaloids are sequestered from their arthropod-rich diet, including ants, beetles, mites and millipedes, and released through their multicellular exocrine glands [7]. Approximately 170 alkaloids and 15 structural classes have already been identified in 9 species of *Melanophryniscus* [8], such as 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, pumiliotoxins, tricyclics, and decahydroquinolines being the most commonly observed [9–11].

Melanophryniscus admirabilis is officially listed as Critically Endangered and is part of the Action Plan for the Conservation of Amphibians and Reptiles in southern Brazil. It is important to highlight that the species' main threats are the ongoing loss of habitat quality resulting from anthropogenic infrastructures and activities such as hydroelectric power plant, deforestation, pesticide use in tobacco and soybean plantations, livestock activity, illegal pet trade, and trampling by tourists in reproductive sites [3]. Due to the vulnerability of the unique known population of this species, the conservation of *M. admirabilis* is a priority in Brazil. The few studies involving this species are most focused on biology and ecology [12], ecotoxicology [13] and so far, no work has been carried out to evaluate the presence of microorganisms in this species. Microbes play an important role in maintaining animal health, in addition, the adaptation and persistence of animal species in impacted environments have been associated with microbiota [2,14]. Microbiota's composition could help promote a greater understanding of species' physiological status and niche divergences under differing environmental conditions [15,16].

The study of composition, diversity, and function of microbial communities not only mirrors the host health species maintenance in the environment but, may also reflect the ecological condition of the habitat. To date, amphibian skin and gut microbiome has been relatively well studied, although studies involving oral microbiome are still scarce [2,17–19]. In this sense, to ensure the successful conservation of *M. admirabilis*, it is important to assess the microbiota of this species. Therefore, this study aims to characterize the oral bacterial community of wild *M. admirabilis* and to address the question of how this community might contribute to toad's adaptation in the anthropogenic environment and its general metabolic capabilities.

2. Materials and Methods

2.1 Sample Collection

Eleven oral samples were collected from wild *Melanophryniscus admirabilis* Fig 1a and 1b (Table 1). Samples were taken from active toads in the breeding sites in the Forqueta river's margins in the Perau de Janeiro locality, Arvorezinha, Rio Grande do Sul, Brazil (52°18''W, 28°51'S). The area is situated at the southern end of the Atlantic Forest, in a transitional phytoecological region between the Mixed Ombrophilous Forest and the Deciduous Seasonal Forest [20].

The oral swab collection was performed according to the sample collection protocol [21]. Oral samples were collected using commercially available sterile cotton-tipped swab sticks. All samples were placed in sterile tubes, kept on ice, and sent to our laboratory for storage at -80^{0}C. The toads were released back into the wild immediately after the sample collection. All specimens are individually marked using a photo identification protocol [12].

97 *2.2 Ethics and sampling permits*

98 This study was carried out following the recommendations of the Chico Mendes Institute for
99 Biodiversity Conservation (ICMBio) and was approved by the Research and Ethics Committees at
100 Federal University of Rio Grande do Sul (Projects 19541, 25526, and 25528). The protocol was approved
101 by the Information and Authorization System in Biodiversity (SISBIO) number 40004-5 and 10341-1 (for
102 M. Borges-Martins). All possible measures were taken to reduce the impact of our sampling protocol,
103 which is part of a larger program intended for the study, monitoring, and conservation of the only
104 known admirable red-belly toad population. Research priorities and protocols are also part of the
105 Action Plan for the Conservation of Amphibians and Reptiles in southern Brazil [12].

106 *2.3 DNA extraction, PCR-amplification of bacterial 16S rRNA genes and sequencing*

107 Total DNA from the oral swabs samples was extracted using DNeasy Blood and Tissue Kit
108 (Qiagen,Valencia, CA, USA) according to the manufacturer's instructions. The DNA concentration was
109 determined using the Qubit, and DNA quality was verified using the NanoDrop ND-1000 (Thermo
110 Fisher Scientific, Waltham, Massachusetts, USA).
111 To characterize the bacterial community present in each oral, fragments of the *16S rRNA* gene were
112 amplified using the primers 515F and 806R [22] and further sequenced using a PGM Ion Torrent
113 (Thermo Fisher Scientific, Waltham, MA, USA). Multiple samples were PCR-amplified using barcoded
114 primers linked with the Ion adapter "A" sequence and Ion adapter "P1" sequence to obtain a sequence
115 of primer composed for A-barcode-806R and P1-515F adapter and primers. PCR reactions were carried
116 out with the Platinum *Taq* DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA). PCR
117 was performed with High Fidelity PCR buffer, 2U of Platinum *Taq* DNA Polymerase, 2 mM of $MgSO_4$,
118 0.2 mM of dNTP Mix, 25 μg of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA), 0.1 μM of each forward
119 primer, approximately 30 ng of DNA template and ultrapure water to complete a final volume of 25 μL
120 per reaction. The PCR conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 56 °C for
121 45 s, and 68 °C for 1 min, and a final extension step of 68 °C for 10 min.
122 Samples were sequenced at the Universidade Federal do Pampa (UNIPAMPA, Bagé, RS, Brazil).
123 After purifying PCR amplicons using Agencount AMPure Beads (Beckman Coulter), library
124 preparation with the Ion OneTouch™ 2 System fitted with the Ion PGMTM OT2 400 Kit Template
125 (Thermo Fisher Scientific, Waltham, MA, USA) from an initial amount of 100 ng of PCR product. Since
126 all samples were sequenced in a multiplexed PGM run, barcode sequences were used to identify each
127 sample from the total sequencing output. Sequencing was conducted on an Ion Personal Genome
128 Machine (PGM) System (Thermo Fisher) using a chip Ion 316, following the manufacturer's
129 instructions. Sequences have been submitted to the EMBL database under accession number
130 PRJEB33232. Despite the short read lengths (~290 bp), this targeted gene region should also provide
131 sufficient resolution.

132 *2.4 Bacterial community analysis*

133 Bioinformatics analysis of *16S rRNA* amplicons was performed using QIIME 2 version 2019.7 [22].
134 Raw sequence data were quality filtered, denoised and chimera filtered using the q2-dada2- plugin with
135 DADA2 pipeline Callahan [23]. The 5′ end 3′ nucleotide bases were trimmed from forward and reverse
136 read sequences due to low quality. Reads with several expected errors higher than 4 were discarded.
137 Read length filtering was applied and the reads were trimmed at the first instance of a quality score less
138 than or equal to 2. The resulting reads were truncated at 200 bp length. Chimera removal was performed
139 using the consensus method. The amplicon sequence variants (ASVs) obtained by DADA2 pipeline
140 were merged into a single feature table using the q2-feature-table plugin.
141 The ASV's were aligned with MAFFT (via q2-alignment) [24] and used to construct a phylogeny
142 with fasttree2 (via q2-phylogeny) [25]. Taxonomy was assigned to ASV's using the q2-feature-classifier
143 [26] classify-sklearn naïve Bayes taxonomy classifier. The classifier was trained using extracted
144 Greengenes 13_8 reference sequences with 99% similarity from *16S rRNA* variable region 4 (V4). The

resulting feature table, rooted tree from reconstructed phylogeny, and taxonomy classification were imported from QIIME2 to R v3.6.1 environment for further data analysis using Microbiome v1.6.0 and Phyloseq v1.28.0 R packages [27]. For Taxonomic analysis, feature table was transformed to compositional data for taxa bar plot composition visualization of the 5 most abundant phylum and families using plot composition function from Microbiome R package.

The taxon diversity study (richness and evenness) within the samples was performed employing the Shannon diversity, the InvSimpson diversity, and the Chao1 index, whereas the observed species metrics calculation and diversity between samples were estimated using Microbiome and Phyloseq packages in R. The significance was estimated with a pairwise comparison using a non-parametric test Wilcoxon [28], using function from Microbiome R package.

2.5 Functional predictions from amplicon sequences

A predictive functional profile of the oral bacterial community was conducted using PICRUSt2 (reference database) QIIME2 plugin. PICRUSt2 output is a biom table with rows as KO terms and samples as columns. The KO terms were mapped into KEGG levels and imported to STAMP (reference database) for statistical analysis. Samples were divided into two gender groups (F-M), and a Welch's t-test was performed to evaluate the significance of functional predictions with p-value < 0.05. Benjamini–Hochberg adjusted p-value was calculated to control the false discovery rate (FDR) in multiple testing. The KEGG groups were considered significantly enriched by satisfying an FDR corrected p-value of 0.05.

3. Results

A total of 181,350 sequences were obtained from the oral samples of wild *Melanophryniscus admirabilis* after discarding (cleaning) the substandard sequences. Among these cleaned sequences, we obtained 13,650 ASVs per sample, which were grouped into 1,039 ASVs. Sequence analysis grouped the reads into16 phyla, 34 classes, 39 orders, and 77 families.

Five phyla presented relative abundance higher than 1% and were present in all samples evaluated. Among them, Proteobacteria dominated the oral microbiota of wild *M. admirabilis*, with the highest relative abundance (53%) followed by Firmicutes (18%), Bacteroidetes (17%), Actinobacteria (5%), and Fusobacteria (2%) (Fig. 2; Table S1). These sequences belonged mainly to 7 orders: Burkholderiales (23%), Bacteroidales (14%), Lactobacillales (8%), Clostridiales (8%), Enterobacteriales (7%) Pseudomonadales (5%), and Actinomycetales (5%) (Table S2).

In addition, 77 families were detected in oral samples; however, only 28 families exhibited a relative average abundance of ≥ 1% (Fig. S1; Table S3). Burkholderiaceae (16%), Prevotellaceae (10%), Enterobacteriaceae (7%), Comamonadaceae (6%), and Streptococcaceae (6%) were more abundant and were shared among all samples evaluated. Alpha did not exhibit any identifiable change in bacteria composition among all oral samples ($p > 0.05$) (Fig. 3).

To better understand the important role of the oral bacterial microbiota present in wild *M. admirabilis*, PICRUSt2 program was used to predict our *16S rRNA* based high-throughput sequencing data. It can be seen that the metabolic functions were enriched in our samples and functional features in 39 pathways have been observed, including membrane transport proteins, amino acids metabolism, carbohydrate metabolism, energy metabolism, replication and repair systems, cofactor and vitamin metabolism, nucleotide metabolism, xenobiotic biodegradation metabolism, lipid metabolism, metabolism of other amino acids, polypeptide and terpenoid metabolism, biosynthesis of other secondary metabolites and others (Fig.S2).

We correlated the microbial functional features with the important pathways associated with toads habitat and diet, such as xenobiotic degradation and metabolism. In total, 16 pathways were identified (Fig. 4; Table S4), being two pathways related to benzoate and toluene degradation with elevated frequency of amplicon sequence variants (average of ASVs = 54486 and 36950, respectively). Data

analysis showed a higher standard deviation for benzoate degradation and toluene degradation when compared to polycyclic aromatic hydrocarbon degradation (Table S5). Other groups of xenobiotic activity are aminobenzoate degradation (average of ASVs= 30067), chloroalkane and chloroalkene degradation (average of ASVs = 25160), drug metabolism-cytochrome P450 (average of ASVs = 19923), naphthalene degradation (average of ASVs = 19789), nitrotoluene degradation (average of ASVs = 12598), ethylbenzene degradation (average of ASVs = 9718), dioxin degradation and biosynthesis (average of ASVs = 6847), atrazine degradation (average of ASVs = 5576) and fluorobenzoate degradation (average of ASVs = 5864) (Table S5).

4. Discussion

Amphibian microbiome studies have been increasing in recent years to facilitate an improved understanding of the diverse communities of bacteria, fungi, and viruses that inhabit their bodies [2,17–19,29,30]. The skin microbiome has been extensively studied due to its relationship to an emergent disease caused by the chytrid fungus (*Batrachochytrium dendrobatidis*) [2,31]. However, knowledge regarding the taxonomic content of amphibian oral microbiota remains extremely limited. Here, we describe, for the first time, using high-throughput sequencing, the bacterial communities present in the oral cavity of wild *M. admirabilis*.

Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (accounted for 95% of the oral microbial community composition), represented typical mucosal taxa and were shared with all samples. These microbial phyla have been associated with symbiotic roles and are commonly observed in the amphibian gastrointestinal tract [31–33]. Chang et al. [30] have reported that Bacteroides, Firmicutes, and Proteobacteria were also dominant in rice frogs' intestinal microbiota (*Fejervarya limnocharis*) in natural and farmland habitats. According to a study in Canada and United States, over 75% of the gut microbial composition of Northern Leopard frogs (*Lithobates pipiens*) were Proteobacteria and Firmicutes [34].

Proteobacteria in oral samples of wild *M. admirabilis* represented 53% of the total phylum. The diet route constitutes an important source of organisms in the oral and gastrointestinal tract of the animals. The presence of Proteobacteria in oral samples may be associated with toads diet arthropod-rich since this phylum was observed as dominant in cuticular microbiomes of ants and gut microbiome of arthropod [35]. On the other hand, the predominance of this phylum may also be associated with the ability to synthesize bioactive secondary metabolites, frequently observed in several bacteria of this phylum [36]. For example, *Janthinobacterium lividum* present in amphibian guts inhibited the growth of lethal amphibian fungi [37]. In addition, antifungal activity from the genus *Pseudomonas* was discovered on the skin of frogs (*Rana muscosa*) and leopard frogs (*Rana pipiens*) [38,39]. In this sense, the oral microbiota of amphibians should be more investigate since it may be a potential source of compounds with antimicrobial activity, impacting their associated microbial communities' diversity or composition.

The oral cavity of wild *M. admirabilis* was dominated by the orders Burkholderiales (Burkholderiaceae and Comamonadaceae), Enterobacteriales (Enterobacteriaceae), and Bacteroidales (Prevotellaceae). Some microorganisms belonging to these orders coexist with frogs and their habitat. For example, Burkholderiales is found in the skins of terrestrial (*Rhinella marina*, *Litoria nasuta*, and *Limnodynases convexiusculus*) and arboreal (*Litoria caerulea*, *Litoria rubella*, and *Litoria rothii*) anuran species [40]. Furthermore, Burkholderiaceae and Comamonadaceae family members have diverse ecological niches, such as soil, water associated with plants, animals, and fungi [41,42]. Moreover, Bacteroidales have been reported as symbiotic bacteria essential for the digestive activity of several organisms [37].

In the present study, we observed similar composition among microbial communities in all oral samples. Recently, Chang et al. [30] hypothesized that the composition of gut microbiota from frogs should be governed by the endogenous gut environment that is shaped by the physical, physiological, and immune properties of host species, and would be less influenced by the surrounding environment. Here, we suggest that the similarity in the oral bacteria community present in the *M. admirabilis* might be also ruled by the endogenous oral environment, such as saliva

fluid [43] and the surrounding environment, since this frog species is microendemic and its diet consists of arthropods (such as Formicidae, Acari, and Coleoptera) that lives around this environment [44].

One of the most striking results by metagenome predictions was the xenobiotic degradation in the bacterial community. The occurrence of communities of bacteria harboring associated with this pathway may suggest positive effects on toads health in anthropogenic pollutants. *Melanophryniscus admirabilis* constitute a threatened Class of vertebrates, and the population declines observed are due to a synergy of different factors, including habitat degradation and fragmentation, mostly for agricultural purposes, and the exposure to contaminants derived from these activities [12]. Nutrient enrichment from agricultural pollution may reshape the structure of the microbiome composition of aquatic animals and increase their vulnerability to disease [2]. A study analyzed possible alterations in metabolic and oxidative parameters of total homogenate in *M. admirabilis* tadpoles exposed to two different concentrations of commercial formulations containing Sulfentrazone (Boral® 500 SC) and two concentrations containing Glyphosate (Roundup® Original). Significant alterations in metabolic and oxidative parameters were observed in groups exposed to Sulfentrazone and Glyphosate herbicides. However, the tadpoles were capable of moderating the potential oxidative lipid damage [13]. Upon analyzing these results in light of the results of the present study, we can suggest that the associated mobilization of enzymes and a microbial community able to be degraded xenobiotics should have a positive effect on the persistence of the *M. admirabilis* in this impacted environment. However, it is important to note that other factors such as habitat fragmentation, UV radiation, and exposure to other pollutants have negative effects that can interact to affect the survival of these animals. Environmental contaminants such as xenobiotics can alter host-associated microbial communities through the displacement of native bacterial taxa by those capable of withstanding chronic exposure to toxic compounds [45]. Finally, improving our knowledge of amphibian microbiomes is important to numerous fields, including species conservation, the detection and quantification of environmental changes and stressors, and the discovery of new compounds with medical applications.

5. Conclusions

Amphibians are important components of most ecosystems and serve a critical role in many food webs, especially in highly diverse tropical areas. *Melanophryniscus admirabilis* is a critically endangered and microendemic species whose survival can be directly related to our ability to understand its ecology, identify the main anthropic impacts, and act to preserve its habitat. This work advances the understanding of the oral microbiota of this species. Our data support the predominance of the phylum Proteobacteria in the oral microbiota of *M. admirabilis*. No significant differences among the microbial community profile from different samples were reported, suggesting that dietary restriction may directly influence the bacterial composition. The oral microbiota contributed to a range of metabolism pathways, with membrane transport, amino acid metabolism, carbohydrate metabolism, replication, and repair predicted as the most prominent categories. The results highlight the potential functional profiles of the xenobiotic degradation pathway in the oral microbiota of these toads. These communities might have important roles in the health and survival of this species in their environment, whereas also serving as an essential component of a successful conservation strategy. Therefore, our results contribute more ecological aspects about oral microbiota of this species that may have important implications for the conservation and management of this critically endangered species since it only occurs in a narrow range of environmental conditions and is experiencing an ongoing loss of habitat quality.

Supplementary Materials: Table S1. Percentage and the average of identified phyla among oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Table S2.** Percentage and average of identified order among oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Table S3.** Percentage and average of identified family among oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Table S4.** Amplicon sequence variants and average of prediction of xenobiotic/drug metabolism identified

among oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Table S5.** Average of amplicon sequence variants and standard deviation of xenobiotic functional prediction in oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Figure S1.** Oral bacterial microbiota composition at the family level in oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads). **Figure S2.** Abundance of predicted genes assigned to Kyoto Encyclopedia of Genes and Genomes – KEGG) categories for metabolism in oral samples of wild *Melanophryniscus admirabilis* (admirable red-belly toads).

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TABLES AND FIGURES

Table 1. Details of wild *Melanophryniscus admirabilis* (admirable red-belly toads) were analyzed in this study.

SAMPLE (ID)	SVL* (mm)	MASS (g)	SEX
AC421	31.51	3.1	Male
AC422	29.88	3	Male
AC423	30.63	3.2	Male
TA01 I	35.46	3.6	Female
TA02 II	33.62	3.2	Male
TA04 IV	31.50	2.7	Male
TA05	34.72	3.8	Female
TA07 VII	32.33	3.6	Male
TA10 X	33.87	4.2	Female
TA11 XI	33.67	4.1	Male
TA12 XII	35.07	3.9	Male

*SVL - Snout–vent length

Fig 1. Wild *Melanophryniscus admirabilis* (admirable red-belly toads) in the Forqueta river's margins in the Perau de Janeiro Arvorezinha, South Brazil. (A) Oral sample collection from the (Photo: Márcio Borges-Martins). (B) *Melanophryniscus admirabilis* in the breeding sites in the Forqueta river's margins in the Perau de Janeiro this environment (Photo: Márcio Borges-Martins).

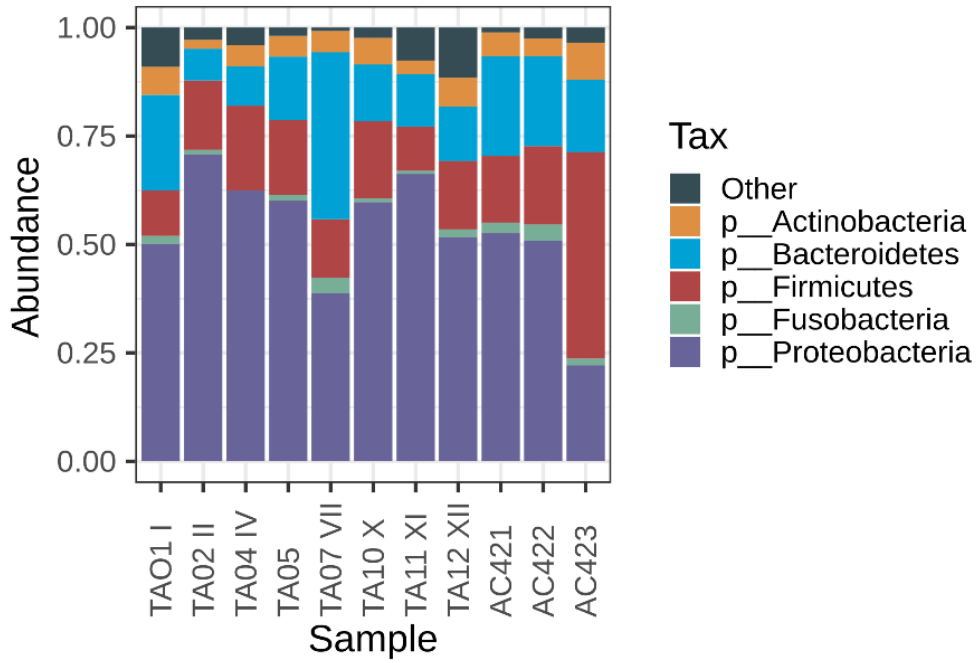

Fig 2. Oral bacterial composition of wild *Melanophryniscus admirabilis* (admirable red-belly toads admirable red-belly toads). Taxonomic composition of the oral microbiota among the eleven samples was compared based on the relative abundance (reads of a taxon/total reads in a sample).

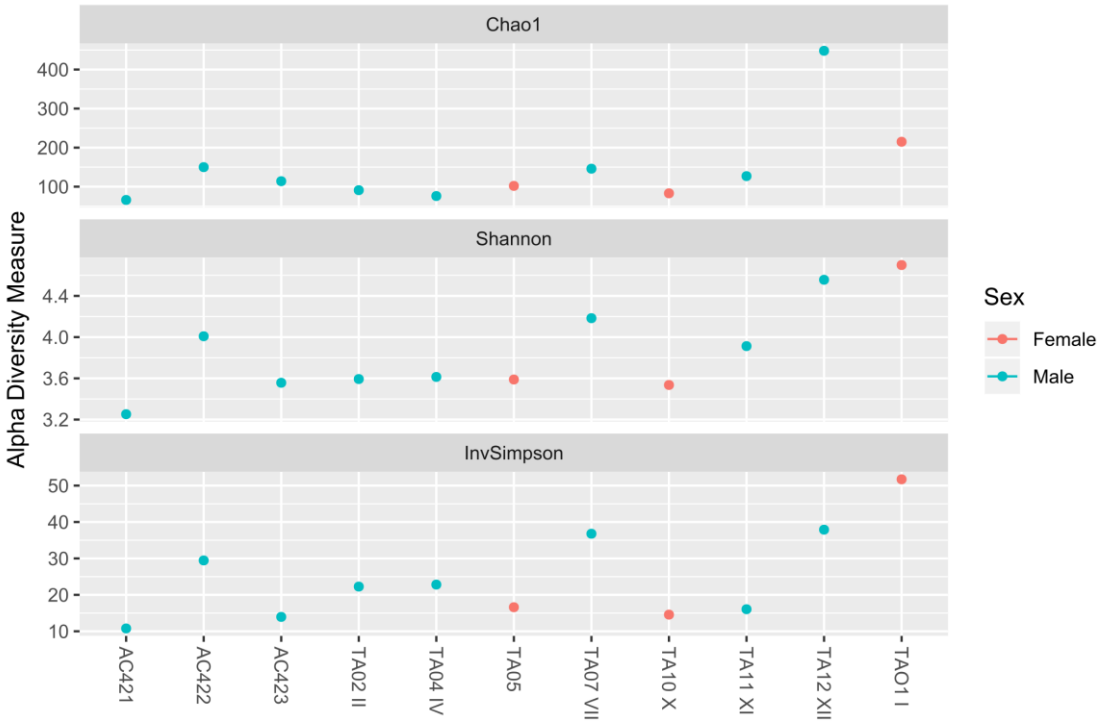

Fig 3. Alpha diversity comparisons of oral bacterial microbiota of wild *Melanophryniscus admirabilis* (admirable red-belly toads). Alpha-diversity analysis based on Chao 1 diversity (A), Shannon diversity (B) and InvSimpson diversity (C), measure of species richness based on amplicon sequence variants (ASVs) of the eleven oral samples collected from wild *Melanophryniscus admirabilis*. No significant difference among the samples was observed.

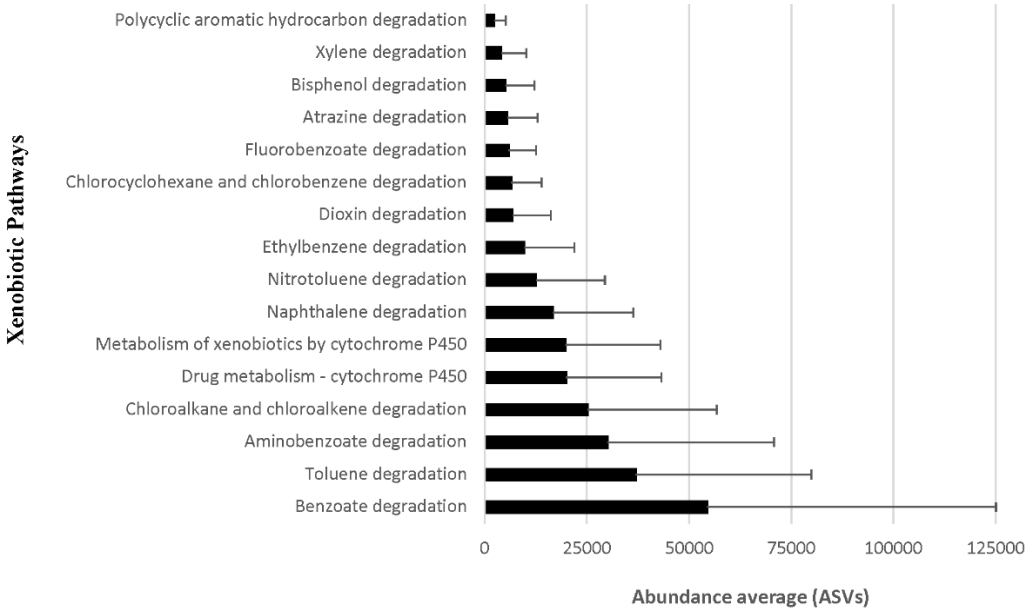

Fig.4. Average relative abundances of prominent (1% average relative abundance) amplicon sequence variants (ASVs) among the *16S rRNA* gene profiles of oral samples from wild *Melanophryniscus admirabilis* belonging to the predicted xenobiotic pathways in relative KEGG Level 2.