

1 **FIRST REPORT OF HALOBACTERIA DOMINANCE IN A**
2 **TROPICAL CAVE MICROBIOME**

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31 **Abstract**

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33 Scarce studies on microbial diversity in tropical caves have been published, a subterranean
34 system still neglected from a microbiological point of view. Although most published studies
35 are about temperate caves, usually archaeas and fungi have less attention than bacterial
36 communities. Here, the microbiome structure and composition in a tropical cave system, as
37 well the main environmental drivers, were studied during the wet and dry season. Soil and
38 sediments from three different habitats at the cave (surface, entrance cave and dark zone) were
39 sampled. Samples were characterized (temperature, air and substrate humidity, salinity, pH,
40 nitrogen and organic carbon content, and chemical composition) and the microbiome was
41 assessed by high-throughput sequencing, using amplicon sequencing (16S and ITS).
42 Prokaryotic communities were dominated by *Halobacteria*, *Actinobacteria* and *Bacilli*, while
43 fungal communities showed high abundance of *Sordariomycetes*. Microbiomes from the cave
44 entrance, where a significantly elevated salinity levels were found, supported up to 63% of
45 *Haloarchaea* compared to the other habitats studied. Differences in community structure were
46 significant between habitats, but no influence of the season was observed. Main environmental
47 drivers of community assembly included nitrogen and organic carbon content, temperature,
48 and salinity. This is the first report of *Halobacteria* dominance in cave habitats, where they
49 likely play important roles in nitrogen and phosphorus cycles. The cave entrance had lower
50 diversity, but higher degree of microbial endemism, which characterize it as an important cave
51 ecotone. The prevalence of heterotrophic microbial groups implies trophic structure based on
52 detritivores, particularly in the dark zones. Our study brings new insights on microbiome
53 composition in the underexplored tropical cave habitats.

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58 1 INTRODUCTION

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60 Caves are generally classified as extreme environments due to the prevailing
61 oligotrophy and lack of light to support photosynthesis. They therefore represent highly
62 specialized ecosystems with no autochthonous photosynthesis-based primary production,
63 dependent mostly on allochthonous carbon inputs [1]. Microbial biodiversity, function, and
64 community dynamics in caves are still considered somewhat of a “black box”. Although the
65 number of published results on cave microbiology has increased over the past decade, most of
66 them were based on temperate cave habitats, and the information on tropical caves are still
67 scarce [2]. Tropical caves are in regions differing significantly in environmental conditions
68 regarding the temperate caves, which include, in general, climate with higher temperatures, a
69 dense tree canopy whole year, due the lack of a dormant period in the winter or dry season, and
70 higher primary production [3]. Such differences can influence the structure and dynamics of
71 microbial communities in caves [4 - 5], highlighting the importance to understand the microbial
72 ecology in tropical cave environments over a seasonal cycle.

73 Even though the published literature covers a wide variety of microbial habitats within
74 the caves such as soils, sediments, stream water, and rock surfaces, most information is limited
75 to describing the *Bacteria* domain only. Indeed, caves show high abundances of
76 *Proteobacteria*, presumably involved in nitrogen fixation, along with significant populations
77 of *Actinobacteria*, with a suggested role in carbon turnover [6]. Nevertheless, studies
78 evaluating the presence and dynamics of other microbial groups in the caves - the *Archaea* and
79 *Eukarya* – are rare. Recently, after a microbiome definition update, researchers have
80 highlighted the importance of considering all microorganisms belonging to different kingdoms
81 (prokaryotes: bacteria, archaea, and micro-eukaryotes: as fungi), as well as their functions and
82 interactions, as part of microbiome studies [7].

83 Next generation sequencing technology advances in the last decade made it easier to
84 assess the uncultured microbial diversity in cave environments and recent studies have
85 demonstrated the presence of *Archaea*, most frequently from the phyla *Thaumarchaeota* and
86 *Euryarchaeota* [8 - 10]. Methanogenic archaea (MA) and ammonia-oxidizing archaea (AOA)
87 are the best-characterized archaeal groups and these microorganisms are targeted in studies of
88 subterranean environments due to their ecological importance in biogeochemical cycling [11].
89 These microorganisms are involved in the terminal steps of carbon flows, nitrification
90 processes [12], primary production [13], and denitrification [14] in terrestrial and aquatic
91 environments. There are currently no *Thaumarchaeota* in culture. Although these are the most
92 frequently encountered *Archaea* in caves, we have very limited information on the possible
93 biogeochemical and ecological roles of *Thaumarchaeota* in these specific environments [11,
94 15 - 16]. Most of what we do know has been inferred from metagenomics studies:
95 *Thaumarchaeota* are likely to play an important role in ammonia oxidation in temperate caves
96 [10]. Some cave microbiomes contain a large proportion of *Euryarchaeota*. For instance, four
97 *Euryarchaeota* classes - *Methanomicrobia*, *Thermoplasmata*, *Halobacteria* and
98 *Methanobacteria* - were found in Indian caves systems [8]. The *Euryarchaeota* include extreme
99 halophiles, sulfate reducers, thermophilic heterotrophs, and methanogens [17].

100 More than 1150 species of fungi, belonging to 550 genera, have already been reported
101 in caves worldwide [18]. The most identified fungi in caves belongs to the phyla Ascomycota,
102 Basidiomycota, and Zygomycota [19]; however, the relevance of these findings should be
103 viewed with caution, as these studies utilize cultivation-dependent techniques. The difficulty
104 of laboratory cultivation, especially those fungi colonizing rocks or low metabolic activity,
105 limits the knowledge of the real diversity, as well as the exploration of the ecological roles
106 performed by these microorganisms in the cave systems. Cave fungi are decomposers or
107 parasites, although they can also aid in speleogenesis processes through the precipitation of

108 secondary minerals [20]. Fungi are the main saprophytic organisms in cave systems and play
109 an important role in the food web [19]. However, pathogenic fungi receive more attention in
110 cave studies due to health and economic concerns, which mainly include *Histoplasma*
111 *capsulatum* and *Pseudogymnoascus destructans*.

112 Taking into account i) the tropical caves are neglected from a microbiological point of
113 view and ii) the gap in the information about archaeas and fungi in cave microbiomes, the main
114 goal of the present study were the analysis the structure and composition of the microbiomes
115 (here including archaea, bacteria and fungi), in different habitats within a tropical cave system,
116 to evaluate the main environmental factors that drive the structure of the microbiomes, and to
117 discuss the possible role of the cave microorganisms in the trophic structure of the subterranean
118 habitats. This is the first research in Brazilian caves using new generation sequencing to assess
119 fungal and prokaryotic diversity. Belonging to the several larger karst areas in Brazil, Terra
120 Ronca State Park (PETeR) is a state conservation unit located in Central Brazil. PETeR harbor
121 the main complex of cave systems in Brazil, including several superficial and subterranean
122 drainages, with great potential for the transport of organic matter, causing accumulations of
123 debris in some caves. This causes high habitat differentiation, accompanied by high richness
124 of subterranean terrestrial and aquatic taxa [21 - 25]. Although the biology of these biodiversity
125 hotspots, especially that of the subterranean fauna, is well known, no studies focused on the
126 microbial assemblages were published so far.

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129 **2 MATERILAS AND METHODS**

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131 **2.1 STUDY SITE**

132 The Terra Ronca State Park (PETeR) (46°100' - 46°300'S; 13°300' – 13°500' W),
133 located within São Domingos city (Goiás State, central Brazil), has a large subterranean system

134 formed by rivers arriving from the Serra Geral Plateau, a morphological feature that originated
135 in the Cretaceous, in the sandstones of the Urucuia Formation [26]. The PETeR is a karst area
136 crossed by parallel streams running westwards to join the Paraná River, a tributary of the Upper
137 Tocantins River, in the Amazonas Basin. The study area belongs to the Cerrado
138 phytogeographical domain (a savanna-like vegetation). The climate is tropical semi-humid
139 with a mean annual precipitation of about 1270 mm yr⁻¹ [27]. The wet season extends from
140 November to April, with rainfall essentially absent between May and October (dry season).
141 Although the area is a Conservation Unit, the park is threatened by anthropogenic impact, such
142 as deforestation for agriculture and uncontrolled tourism, and the springs of the main rivers are
143 outside the boundary of the Conservation Unit, a place used for cattle, crops, consequently
144 silting and polluting rivers and affecting the cave systems [28].

145 The Lapa da Terra Ronca I cave (TR cave) is a part of the Terra Ronca-Malhada cave
146 system (Fig 1, Fig S1). The cave has an entrance of ca 100 meters high and 120 meters wide,
147 with a built altar measuring 760 meters long and 100 meters high where the local religious
148 ceremony of “Bom Jesus da Lapa” takes place at the beginning of August.

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150 **2.2 SAMPLING**

151 Samplings were conducted in April 2016 (wet season) and October 2016 (dry season)
152 (license n° 28992-11/ICMBio/SISBIO and n° 14886/2010/Secima, Goiás). One square of
153 approximately 0.25 m² was sampled in three distinct habitats of the cave: surface (cave exterior
154 – around 50 meters far from cave entrance), cave entrance and the dark zone (no sunlight). All
155 the different habitats were sampled in replicates. Approximately 300 g of substrate (soil or cave
156 sediment, up to 10 cm in depth) were collected at five different points of the square area at each
157 site forming a composite sample. Samples were collected with the aid of a shovel and stored in

158 sterile plastic bags. The samples were transported to the laboratory in coolers, homogenized,
159 sieved (2 mm mesh) and stored in the refrigerator at 4 °C.

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161 **2.3 PHYSICAL AND CHEMICAL PARAMETERS**

162 Temperature (°C), air humidity (%) and luminosity (Lux) were measured at each
163 sampled area with a minimum time interval of 1 minute between measurements (Thermo-
164 hygrometer Instruntherm THAL-300, 0.1 resolution and $\pm 5.0\%$ accuracy). Substrate pH was
165 measured at the substrate:water ratio of 1:2.5 (weight/weight) and substrate salinity was
166 estimated using a refractometer. The moisture in the cave sediment and soil samples was
167 estimated by the gravimetric method with drying at 105 °C for 20 h to 7 days after sampling,
168 and the results expressed as dry weight percentage. Organic carbon (OC) concentrations were
169 measured colorimetrically using the method of [29]. Total nitrogen content (TN) was
170 determined by Kjeldahl digestion followed by ammonia distillation (indophenol blue method)
171 [30]. The composition of the subterranean substrate was assessed by scanning electron
172 microscopy (SEM), together with chemical analysis by energy dispersive spectroscopy (EDS).
173 An Oxford EDS coupled to a FEI Quanta 250 SEM was used to examine the chemical
174 composition of the samples. The substrate was adhered to a double-sided copper tape mounted
175 on an aluminum stub for observation [31]. Fifteen ESEM images and corresponding EDS
176 spectra of elements were acquired for each sample on average.

177

178 **2.4 DNA EXTRACTION AND SEQUENCING**

179 DNA was extracted from 0.25 g of each homogenized sample using the MoBio
180 PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) following
181 manufacturer's instructions. The quality and quantity of extracted DNA were verified by the
182 examining products on TBE agarose gels and by measuring the ratio of absorbance at 260 and

183 280 nm, and 260 and 230 nm, with a Thermo Scientific Nanodrop 2000c Spectrophotometer.
184 For prokaryotes, the V3–V4 region of the 16S rDNA genes was amplified using the primer
185 pair 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-
186 GACTACHVGGGTATCTAATCC-3'). The ITS1 (5'-GCATCGATGAAGAACGCAGC-3') /
187 ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were used to assess the diversity of fungi
188 [32 - 33]. Briefly, for a 25µL PCR reaction with 8.5 µl of Kapa High-fidelity HOTSTART
189 ready MIX, 0.1 µM of each primer, 10 µl of PCR-grade water, and 10 ng of DNA extract were
190 used. The 16S rDNA amplification conditions were 95°C for 3 min, followed by 35 cycles at
191 95 °C for 30 s, 55 °C for 1 min 15 s, 72°C for 45 s, and finally 72°C for 5 min. The ITS
192 amplification had an initial stage of 95 °C for 3 min, 35 cycles of 95 °C for 15 s, 60 °C for 15
193 s, 72 °C for 45 s, and finally 72 °C for 10 min. PCR products were purified with the AMPURE
194 XP magnetic bead kit (Bechman Coulter) and indexed with the Nextera XT kit V2 (Illumina)
195 to separate samples for sequencing. A second step of purification with magnetic beads
196 followed, and then the metagenomic pool was assembled with 5 µl of each library. High-
197 throughput sequencing of the V3-V4 and ITS regions was performed using the Illumina MiSeq
198 sequencing platform. ITS amplicons were sequenced at Macrogen (Macrogen Inc., Seoul,
199 Korea), while the 16S samples at the Multi-user Laboratory of Sequencing in Large Scale and
200 Gene Expression (São Paulo State University "Julio de Mesquita Filho"). Negative extraction
201 and PCR controls were sequenced together with amplicons samples, and the raw sequences
202 have been deposited in the NCBI Sequence Read Archive (SRA) under project accession
203 number PRJNA723998 (16S) and PRJNA724003 (ITS).

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205 **2.5 BIOINFORMATIC ANALYSIS AND BIODIVERSITY ASSESMENT**

206 Sequencing data were processed using UPARSE [34] in a pipeline internally
207 implemented for 16S and ITS [35]. Paired-end reads were merged with PEAR [36]. Sequences

208 were quality controlled with the following steps: all sequences shorter than 100 pb were
209 discarded, followed by quality dereplication checking, OTU clustering (UPARSE algorithm,
210 similarity $\geq 97\%$), and filtering of chimeras with USEARCH [37 - 38]. Taxonomic
211 classification was performed through BLASTn using the databases SILVA 119.1 for 16S and
212 UNITE for ITS.

213 All statistical analyses were carried out in R Software (R. Core Team, 2016).
214 Environmental variables were analyzed using basic descriptive statistic (Shapiro-Wilks).
215 Analysis of variance and Student t-tests with 5% probability threshold were also applied to
216 verify the significance of the differences among the results. Statistical analyses of microbial
217 community richness, alpha diversity (Shannon index) and community structure were estimated
218 using R *microbiome* package [39]. The distance matrices of community composition were
219 obtained using Bray-Curtis distance and, for environmental matrices, Euclidean distance was
220 used. Relationship between environmental and microbiomes matrices were evaluated by
221 Canonical Correspondence Analysis (CCA) using tools from a number of other R extensions,
222 including *vegan* [40] and *ggplot2* [41]. Differences among sites sampled and the two seasons
223 were tested using permutational multivariate analysis of variance (PERMANOVA) with Bray-
224 Curtis distance, performing 9999 permutations using the *adonis* function [40]. Principal
225 coordinate Analysis (PCoA) ordination using weighted Unifrac was used to evaluate the β -
226 diversity among the sampled sites and a heatmap associated with cluster analyzes (*pheatmap*
227 function) to identify biomarkes for each habitat [42].

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233 3 RESULTS

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235 3.1 PHYSICOCHEMICAL PARAMETERS

236 A summary of all analyzed environmental variables is presented in Table 1. The two
237 sampling seasons differed significantly in mean temperatures and air humidity. Salinity was
238 significantly higher at the cave entrance compared to the other sampling sites, with the
239 difference most pronounced in the dry season (4.42%, compared to 2.36% in the wet season).
240 The substrate in all samples were slightly alkaline (pH ranged between 7.49 and 8.88), and
241 there was a marked trophic gradient present in both (wet and dry) sampling season: higher
242 organic carbon content at the cave entrance (1008.20 mgC kg⁻¹ and 1170.71mgC kg⁻¹,
243 respectively), followed by the surface (820.37mgC kg⁻¹ and 824.48mgC kg⁻¹), and the dark
244 zone site with lowest measured organic carbon concentrations (448.93mgC kg⁻¹ and 334.98
245 mgC kg⁻¹). The surface showed higher amounts of TN in the dry season (0.075mgN kg⁻¹), while
246 higher amounts of TN at the entrance and dark zone sites were observed in the wet season
247 (0.078 mgN kg⁻¹ and 0.036 mgN kg⁻¹, respectively). The detailed analysis of sample chemical
248 composition revealed that silica (Si) was the dominant element. The cave entrance exhibited
249 higher concentration of other essential elements, such as magnesium (Mg) and calcium (Ca)
250 (Table 2). Samples from there were also the only ones where chlorine (Cl), sulfur (S), and
251 phosphorus (P) were present in detectable quantities.

252

253 3.2 MICROBIOME COMPOSITION

254 A total of 12 samples from three different habitats (surface, entrance, and dark zone) at
255 TR cave were sequenced by Illumina MiSeq platform, resulting in 1,156,899 sequences for
256 prokaryotic communities. After contig assembly, trimming, and chimera removal a total of
257 558,322 valid reads were obtained. Representative sequences of 3,517 OTUs were

258 taxonomically annotated from phylum to genus levels (S2 Appendix). The *archaea* and
259 *bacteria* domains showed different relative proportions at the three sampling sites. Taking into
260 account both seasons, the entrance site showed an unusually close proportions of the two
261 domains (44.06% *archaea* and 55.94% *bacteria*), differing significantly in this respect from
262 the dark zone (6.62% *archaea* and 93.38% *bacteria*) and the surface (0.68% *archaea* and
263 99.32% *bacteria*). Particularly the cave entrance showed a larger archaea community in the dry
264 season (75.49% *archaea* and 24.51% *bacteria*) than in the wet season (30.61% *archaea* and
265 69.39% *bacteria*). A total of 4 archaea phyla and 31 bacterial phyla were detected in TR cave
266 samples. *Halobacteria* was the dominant class in archaeal communities, with *Halalkalicoccus*
267 (54.52%) and *Halococcus* (29.46%) as the most representative taxa. *Actinobacteria* were
268 dominant at the surface and dark zone sites, while *Bacilli* was the dominant bacterial class at
269 the cave entrance. Fig 2A shows the main prokaryotic classes found at the sampling sites.
270 Uncultured prokaryotic taxa comprised 24.22% at the genus level. A total of 32.5% of
271 prokaryotic OTUs was shared between the sites, with surface showing a higher proportion of
272 unique OTUs (16.6%), followed by the cave entrance (15.4%) and the dark zone (3.5%). About
273 64.4% of the prokaryotic OTUs were present in both sampling seasons.

274 Regarding fungal communities, a total of 990,619 valid reads and 1,922 OTUs were
275 annotated from phylum to specie levels (S3 Appendix). The mean fungal OTU numbers in
276 three different habitats ranged from 89,340 reads (surface) to 105,820 reads (entrance), with
277 3% cutoff. In total, 415 known genera, 193 known families, 76 known orders and 30 known
278 classes in 7 fungal phyla were detected in the TR cave samples. *Ascomycota* was the most
279 dominant phylum, with the relative abundance of 77.54%, followed by *Basidiomycota* (8.21%),
280 *Chytridiomycota* (0.37%), *Mortierellomycota* (0.36%), *Mucoromycota* (0.01%),
281 *Glomeromycota* (0.002%), and *Rozellomycota* (0.001%). The relative abundance of 10 most
282 detected fungal taxa at the class level for all samples is showed in Fig 2B. The dataset contained

283 fungal sequences unidentifiable at the phylum and class level (13.49% and 14.37%,
284 respectively). *Chaetomium murorum* (17.76%) and *Aspergillus fumigatus* (15.85%) were the
285 most abundant fungal taxa at the surface and entrance, respectively. The dominant taxon at the
286 dark zone site was unidentified at phylum level (11.73%) and was followed by an *Aspergillus*
287 sp. (SH186265.07FU) (6.61%). Only 3.4% OTUs were present in all sampled habitats, with
288 higher proportion of unique OTUs at the cave entrance (42.6%) and dark zone (25.9%). About
289 17.1% of the fungal OTUs were present in both seasons, while 45.6% and 37.3% of the OTUs
290 were unique in the wet and dry season samples, respectively.

291

292 **3.3 MICROBIOME STRUCTURE AND ENVIRONMENTAL DRIVERS**

293 The microbiome structure varied significantly among the different habitats
294 (PERMANOVA, $F_2 = 1.59$, $p = 0.001$), with no effect of the season (PERMANOVA, $F_2 =$
295 0.70 , $p = 0.94$). The same pattern can be observed analyzing only prokaryotic (PERMANOVA,
296 $F_2 = 1.63$, $p = 0.02$) or fungal (PERMANOVA, $F_2 = 1.34$, $p = 0.004$) communities from the
297 sampling sites. Based on these results, the season factor was excluded from the following
298 analyses and the microbiome was considered as a whole, i.e. containing both prokaryotic and
299 fungal assemblages.

300 Species richness (Fig 3) was highest in the surface samples ($1,791.77 \pm 287.24$),
301 followed by the dark zone ($1,627.45 \pm 1,101.80$) and entrance samples ($1,541.22 \pm 478.32$). In
302 contrast, the Shannon index indicated that dark zones (4.38 ± 0.67) and surface (4.32 ± 0.69)
303 had higher microbial diversity than the cave entrance (3.86 ± 0.87). Surface samples showed a
304 slightly increased Simpson dominance (0.08 ± 0.08) compared to the entrance (0.07 ± 0.03)
305 and dark zone (0.04 ± 0.02) samples. A cluster analysis of OTU-level diversity and PCoA based
306 on weighted Unifrac results both showed distinct grouping of samples according to the different
307 cave habitats, with the cave entrance cluster falling between the surface and the dark zone (Fig

308 4A). Overall, the core assemblage associated with each cave habitat showed a degree of
309 specificity (heatmap analysis, Fig 5): *Cladosporium sphaerospermum* (Dothideomycetes) and
310 *Torula* sp. (Saccharomycetes) were characteristic for surface samples, *Aspergillus* sp.
311 (Eurotiomycetes) and *Bovista aestivalis* (Agaricomycetes) for the dark zone, while the cave
312 entrance samples contained up to eight highly specific OTUs including *Rubrobacter* sp.
313 (Rubrobacteria), *Halalkalicoccus tibetensis* (Halobacteria), *Cladosporium* sp.
314 (Dothideomycetes), *Aspergillus* sp. (Eurotiomycetes), *Cladosporium halotolerans*
315 (Dothideomycetes), *Capnodiales* sp. (Dothideomycetes), *Halalkalicoccus* sp. and *Halococcus*
316 *sediminicola* (Halobacteria).

317 Canonical Correspondence Analysis (CCA) was used to identify possible relationships
318 between microbial assemblages and local physicochemical variables at sampling sites (Fig 4B).
319 The analysis of variance using distance matrices was used to find the best set of environmental
320 variables that describe the community structure for each site sampled ($p < 0.05$). Among the
321 tested variables, nitrogen ($F_2 = 1.96$, $p = 0.01$), temperature ($F_2 = 1.49$, $p = 0.01$), organic
322 carbon ($F_2 = 2.02$, $p = 0.01$), and salinity ($F_2 = 1.34$, $p = 0.02$) were the main environmental
323 drivers affecting the distribution of microorganisms. The variables showed a positive
324 correlation with microbiomes at the cave entrance, while the surface and dark zone sites
325 showed an opposite trend. These results clearly show a trophic and salinity gradient affecting
326 the microbial structures at the cave entrance in relation to the other habitats.

327

328 **4 DISCUSSION**

329

330 Caves are widely considered as extreme oligotrophic environments ($<5.0 \text{ mg C L}^{-1}$),
331 although this is largely based on research results on temperate cave environments. However,
332 this assumption of oligotrophic conditions only holds true for subterranean environments that
333 are relatively isolated from the surface, thus having very limited organic input [43]. The TR

334 cave entrance and dark zone sites are relatively well connected with the surface environment,
335 which is a rich source of organic matter as it is characterized by dense vegetation cover. This
336 is reflected in the concentrations of organic carbon, which seems to accumulate at the cave
337 entrance, with its concentration at dark zone site high, in the range of 100 mg g⁻¹. This supports
338 the conclusions of recent research that although both temperate and tropical cave communities
339 are supported by organic inputs from allochthonous sources, and both types of systems show a
340 trophic gradient from surface to dark zones, the tropical subterranean environments are not
341 limited in energy input to the same degree as their temperate counterparts, due to the diversity
342 and abundance of vegetation and its presence throughout the whole year [3, 5]. The
343 environmental differences between tropical and temperate caves directly influenced the
344 microbial communities within subterranean system. Tropical caves are a particularly
345 understudied environment, and most of what we know about microbial communities was based
346 on studies using culture-dependent methods [44 - 47]. While there are some published results
347 generated by culture-independent approaches [6, 48], this is the first comprehensive study
348 reporting the microbiome composition from a tropical cave, including prokaryotic and fungal
349 assemblages through next generation sequencing.

350 TR cave sites hosted a heterotrophic microbiome and most of the dominant phyla
351 identified in this study were previously also found in other cave systems worldwide
352 (summarized in [2]). *Actinobacteria* and *Sordariomycetes* were the dominant classes at the dark
353 zone site, which seems to be a general trend in limestone caves [4, 49]. *Actinobacteria* are
354 known as typical heterotrophs, that play a crucial role in the carbon cycling through organic
355 matter decomposition [50], and good competitors due to their wide metabolic capabilities
356 which enable them to grow on organic compounds of varying complexity and the ability to
357 produce several secondary metabolites, such as antibiotics [51, 52]. *Sordariomycetes*
358 (Ascomycota), although their diversity remains underestimated, represent a recurrent fungal

359 class in caves which also likely play an important role in the decomposition of organic matter,
360 with their biomass (spores and mycelia) as an essential food source for many cave arthropod
361 communities, such as isopods, collembolans, and protozoa [19, 20]. These saprophytic fungi
362 show an aggressive colonization strategy, capable of surviving in extreme environmental
363 conditions, and some fungi of this class, such as *Penicillium* and *Aspergillus*, are able to
364 degrade rocks and solubilize minerals [53]. All these features regarding to cave microbiome
365 are an important advantage to survive in low carbon and nutrient environments, as in dark zone
366 at TR cave. Fungi are often studied in caves because to the concern in detecting pathogenic
367 fungi. *Histoplasma capsulatum*, the main pathogenic fungus in caves, was not found in our
368 results. However, caves can present opportunistic fungi, which can be pathogenic under
369 specific conditions, such as high concentrations of spores and dry air. Fungi species responsible
370 for opportunistic infections have increased in recent decades [54]. *Aspergillus fumigatus*, for
371 instance, was found in all sampled habitats and high abundance in entrance at the TR cave.
372 *Aspergillus fumigatus*, an opportunistic fungus, is the main responsible for aspergillosis and it
373 is necessary to inhale a high load of spores to develop the disease. Spores inhaled by healthy
374 people are eliminated quickly by the immune system or just a weak allergy is developed. On
375 the other hand, people with compromised immune systems can develop a serious pulmonary
376 infection by inhaling a large load of spores [55]. Periodic monitoring of spore loads in TR cave
377 is highly recommended in order to prevent people from entering the cave during periods with
378 a high concentration of spores in the air, especially in drier periods. The use of cave for tourism
379 and scientific purposes with no proper monitoring can lead to health problems for tourists, tour
380 guides and researchers.

381 The chemical composition of the sediment in the TR cave shows the presence of
382 cosmotropic and chaotropic salts. Cosmotropic salts are stabilizing salts, such as NaCl and
383 KCl, that stabilize proteins, whereas chaotropic salts are recognized as destabilizing salts like

384 MgCl₂ that unfold proteins and increase solubility of hydrophobic chemicals [56], which makes
385 the environment inhospitable for many microorganisms. Thus, the salinity can affect microbial
386 communities through the osmotic effect and specific ion effects [57]. The salinity was found
387 to be significantly higher at the cave entrance. Besides being a subterranean biodiversity
388 hotspot, TR cave also has a cultural and religious value for the population from cities around
389 the State Park of Terra Ronca. Since 1920, an annual religious festival attracts thousands of
390 people to the cave entrance in August (dry season), and during this period the soil and sediment
391 are highly compacted. In addition, fireworks containing residues rich in sulphur, potassium,
392 and magnesium are used in large quantities [58]. Therefore, there are three factors possibly
393 contributing to the increased salinity at this particular site: i) intense solar radiation at the cave
394 entrance in comparison to the other two studied habitats and the consequent increase in water
395 evaporation from the sediment; ii) sediment compaction during the religious festival decreases
396 water retention and increases salt concentration in the substrate; and iii) intense and prolonged
397 use of fireworks release salt-containing residues which accumulate in the sediment. All of these
398 factors likely had an important effect on the microbiome composition and supported the
399 dominance of halo-tolerant microorganisms at this site.

400 A significantly higher proportion of archaea, which surprisingly exceeded bacteria in
401 relative abundance during the dry season, was found at the cave entrance site. The few research
402 that has assessed archaeal diversity in caves emphasize the ecological importance of this group
403 in subterranean environments due to their involvement in biogeochemical cycling, especially
404 nitrogen and phosphorus [6, 8, 10]. Organisms belonging to the class *Halobacteria*
405 (*Euryarchaeota*) were the dominant group here, being commonly found in environments with
406 salinity higher than 3% and preference for neutral to alkaline pH [59], have an important role
407 on nitrogen cycling – reducing nitrate and growing by denitrification [60], and hydrolyse
408 insoluble phosphorus compounds to soluble compounds that can easily be assimilated by other

409 organisms [61]. They are facultative phototrophic due the presence of an integral membrane
410 protein known as bacteriorhodopsin [60, 62], a light-driven proton pump converting light
411 energy into chemical energy, which may aid the growth under anoxic conditions, such as in a
412 compacted sediment. All of these features highlighted the survival advantages of the
413 *Halobacteria* at the cave entrance: presence of detectable inorganic phosphorus, under osmotic
414 stress and anthropogenic disturbance.

415 Within the bacteria domain, *Bacilli* was the dominant class at the cave entrance,
416 similarly to the prevalence reported from other cave systems, namely the Ozark cave, USA
417 [51], and Brazilian caves [48]. Interestingly, in the Ararat Plain (Armenia), which represent a
418 hydromorphic saline–alkaline soils, resembling those at the entrance of TR cave, the bacterial
419 community was reported to be highly reduced, almost limited solely to *Bacilli*, which are able
420 to remain viable and growing without competition from other bacteria [63]. Under such
421 conditions, *Bacilli* show a specific survival strategy which includes the synthesis of special
422 desiccation-resistant proteins, the accumulation of non-reducing sugars and the formation of
423 dormant life stages (endospores). In terms of fungi, *Cladosporium halotolerans*, known as
424 black fungi, also showed high prevalence at the cave entrance and this result is likewise
425 consistent with the measured environmental conditions. This organism has been isolated from
426 mine water in the Iron Quadrangle region (Minas Gerais, Brazil), is well adapted to harsh
427 oligotrophic habitats on the surface and subsurface of rocks [64], high radiation, low water
428 availability, long periods of desiccation, and shows high potential for removing Mn from the
429 environment [65]. Thus, the microbiome at the cave entrance clearly shows the highest degree
430 of microbial specialisation compared to the two other studied sites.

431 Microbiome composition and environmental parameters in TR cave clearly showed a
432 distinction among the studied habitats. Canonical Correspondence Analysis (CCA) indicated
433 organic carbon, nitrogen, temperature, and salinity as the main environmental drivers in the

434 structure of microbial communities. Salinity was strongly related to the structure of the
435 microbiome at the entrance cave, as previously discussed, and the others environmental drivers
436 were related to dominant saprophytic microorganisms at surface and dark zone. Previous study
437 at TR cave revealed the availability of carbon and nitrogen influenced the microbial strategies
438 for organic matter decomposition and incorporation of those compounds into their biomass [5].
439 Now our results also support these environmental factors also influence the composition and
440 structure of the microbial communities. The entrance of TR cave can be considered as an
441 ecotone, the transitional zone between adjacent ecological systems (surface and dark zone),
442 where the environment rapidly shifts from one type to another based on abiotic and/or biotic
443 features. Even though many researchers consider ecotone an area with greater richness and
444 diversity than each one of the systems, an ecotone can also support lower diversity if resources
445 vary widely within it or if it is in an area under the influence of severe disturbances [66]. The
446 anthropogenic impact and the unique habitat conditions, such high salinity and solar radiation,
447 at the entrance of TR cave can promote the development of high endemism and dominance of
448 few species, as already seen for invertebrate community in caves [67].

449 In summary, this study is the first to assess the microbiome structure in different
450 habitats of a tropical cave system using high-throughput amplicon sequencing. The
451 microbiomes at the surface and dark zone are composed mainly of heterotrophs
452 microorganisms. This composition together with the relatively high organic carbon
453 concentrations indicate the presence of a trophic network based almost entirely on detritivory.
454 The influence of carbon and nitrogen, as seen in previous studies in TR cave, along with
455 temperature, highlights those as the main drivers on the decomposing microorganisms,
456 especially in the dark zone. Our study also shows for the first time the dominance of Haloarchaea
457 in a limestone cave, which may have an important ecological role in this environment as a
458 phototrophic, phosphate solubilizing archaea, and nitrogen cycle players. Furthermore, these

459 results show that anthropogenic changes can have profound implications for cave soil
460 composition, microbiome structure, and, hence ecosystem functioning, should be considered
461 in the future studies, alongside the commonly researched effects on micro- and
462 macroinvertebrates and vertebrates.

463

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465

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480

481 **6 Reference**

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666 7 Supporting information

667 **Fig S1.** Detailed map of the Terra Ronca - Malhada subterranean system. Highlight for the (A)
668 Terra Ronca I (TR cave) and (B) Terra Ronca II caves. Map: Bambuí Speleological Research
669 Group – GBPE.

670 **S2 Appendix.** Prokaryotical OTU (Operational Taxonomic Unit) table.

671 **S3 Appendix.** Fungal OTU (Operational Taxonomic Unit) table.

672

673 8 FIGURES

674

675 **Fig 1.** Map of Lapa de Terra Ronca I cave (TR cave) located in a Brazil Central region, and
676 the photos shows the surface (A), external (B) and internal (C) view of entrance area and the
677 dark zone (D and E). The dots show the replicates samples in the map at entrance cave (green)
678 and dark zone (red). Surface samples were collected 50 meters far from cave entrance (map:
679 Grupo Bambuí de Pesquisas Espeleológicas – GBPE).

680 **Fig 2.** Relative abundance of prokaryotic (A) and fungal (B) communities at the Class level in
681 TR cave during wet (April / 2016) and dry (October / 2016) season. Only the 10 most abundant
682 classes were shown, which less abundant classes being grouped into "Others".

683 **Fig 3.** Richness (Chao1), Diversity (Shannon) and Dominance (Simpson) indices of microbial
684 communities in three different habitats (surface, entrance and dark zone) at TR cave.

685 **Fig 4.** (A) Principal Coordinate Analysis (PCoA) plot based on weighted Unifrac results and
686 (B) Canonical Correspondence Analysis (CCA) of microbiome data and environmental factors
687 among the whole cave ecosystem, using Bray-Curtis distance and 999 permutations. Only
688 environmental factors with p-values < 0.05 are marked at the graph. Samples from surface,
689 entrance and dark zones are highlighted in blue, green, and orange, respectively.

690 **Fig 5.** Heatmap of microbiome taxons with relative abundance $\geq 1\%$ (core microbiome) from
691 different habitats in the TR cave. Samples from surface, entrance and dark zones are
692 highlighted in blue, green, and red, respectively. Fungal taxa are highlighted in dark blue, while
693 bacteria are grey, and archaea are purple.

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701 **9 TABLES**

702 **Table 1.** Mean values and standard deviations of physical (substrate moisture, air temperature, air humidity and luminosity) and chemical (pH, salinity, organic
703 carbon (OC) and nitrogen (N)) in TR cave on the surface, entrance and subterranean sample sites during wet (April 2016) and dry (October 2016) seasons.

ENVIRONMENTAL PARAMETERS	Season	Surface	Entrance	Dark zone
Substrate moisture (%)	wet	5.60 ± 3.85 ^a	4.03 ± 2.54 ^a	3.39 ± 1.99 ^a
	dry	1.13 ± 0.56 ^a	5.86 ± 1.91 ^b	5.91 ± 1.32 ^b
Temperature (°C)	wet	26.85 ± 0.53	28.33 ± 0.55	26.63 ± 0.98
	dry	29.25 ± 0.10	29.25 ± 0.10	27.38 ± 0.21
Air humidity (%)	wet	73.01 ± 4.24	66.23 ± 0.97	76.71 ± 13.96
	dry	68.86 ± 0.95	64.81 ± 3.93	68.11 ± 1.66
Luminosity (Lux)	wet	1835.33 ± 71.8	43.16 ± 9.3	0
	dry	879.6 ± 161.6	233.8 ± 57.3	0
pH	wet	8.88 ± 0.14	7.49 ± 1.58	7.69 ± 0.25
	dry	8.69 ± 0.29	7.98 ± 0.75	7.59 ± 0.10
Salinity (%)	wet	0.067 ± 0.007	2.366 ± 0.070	0.020 ± 0.001
	dry	0.227 ± 0.007	4.426 ± 0.459	0.111 ± 0.014
OC (mg kg ⁻¹)	wet	820.37 ± 71.61	1008.20 ± 700.86	448.93 ± 145.67
	dry	824.48 ± 700.84	1170.71 ± 1044.51	334.98 ± 51.23
N (mg kg ⁻¹)	wet	0.055 ± 0.040	0.078 ± 0.059	0.036 ± 0.004
	dry	0.075 ± 0.047	0.025 ± 0.002	0.025 ± 0.007

704 **Note:** Different letters in a row means significant difference ($p < 0.05$) between.

705 **Table 2.** Mean values and standard deviations of chemical composition of the substrate (wt.%) in TR cave on surface, entrance and dark sites during wet
706 (April/2016) and dry (October/2016) season.

Sample Sites	Season	O	Si	Al	Fe	Cu	K	Mg	Ca	Cl	S	P
Surface	wet	58.9 ± 3.1	18.1 ± 3.5	3.2 ± 1.6	1.7 ± 0.8	0.8 ± 0.4	0.6 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	NA	NA	NA
	dry	51.9 ± 2.2	28.4 ± 5.6	1.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.2 ± 0.09	0.2 ± 0.05	0.07 ± 0.05	NA	NA	NA
Entrance	wet	58.1 ± 5.2	35.0 ± 1.2	5.4 ± 2.7	2.5 ± 1.3	1.3 ± 0.1	0.4 ± 0.2	17.3 ± 5.4	1.9 ± 2.1	0.04 ± 0.1	0.2 ± 0.1	NA
	dry	60.5 ± 0.7	30.5 ± 9.7	4.4 ± 2.2	2.0 ± 1.2	1.3 ± 0.8	0.9 ± 0.3	12.5 ± 5.6	3.9 ± 0.9	0.04 ± 0.1	0.9 ± 0.5	1.5 ± 0.3
Dark	wet	56.3 ± 3.6	22.1 ± 6.2	2.1 ± 1.1	1.1 ± 0.6	1.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	NA	NA	NA
	dry	59.9 ± 4.3	28.5 ± 7.6	1.6 ± 0.4	0.9 ± 0.5	0.7 ± 0.6	0.07 ± 0.1	NA	0.1 ± 0.1	NA	NA	NA

707

708 **10 Data Availability**

709 Raw sequence data from this study can be downloaded from National Center for Biotechnology
710 Information (NCBI) Sequence Read Archive (SRA) with accession number PRJNA723998
711 (16S) and PRJNA724003 (ITS).

712

713 **11 Conflict of Interest**

714 The authors declare that they have no conflict of interest.

715

716 **12 Author Contributions**

717 PAULA, CCP conceived and designed the study, responsible for chemical and molecular
718 analyzes and wrote the paper with input from all authors. SELEGHIM, MER was in charge of
719 the funding budgets and wrote the paper. BICHUETTE, ME contributed to data analyses,
720 coordinated the field sampling program and contributed to writing of the paper. SIROVÁ, D
721 and SARMENTO, H contributed to data analyses and writing of the paper. FERNANDES, CC
722 and KISHI, LT were involved in the sequencing of the 16S samples.

723

724 **13 Funding**

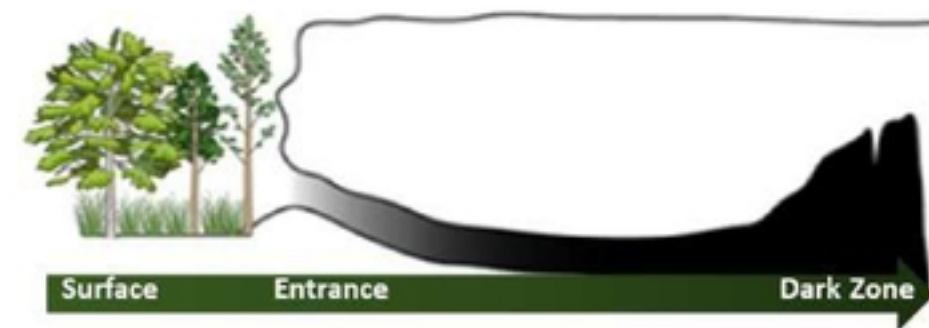
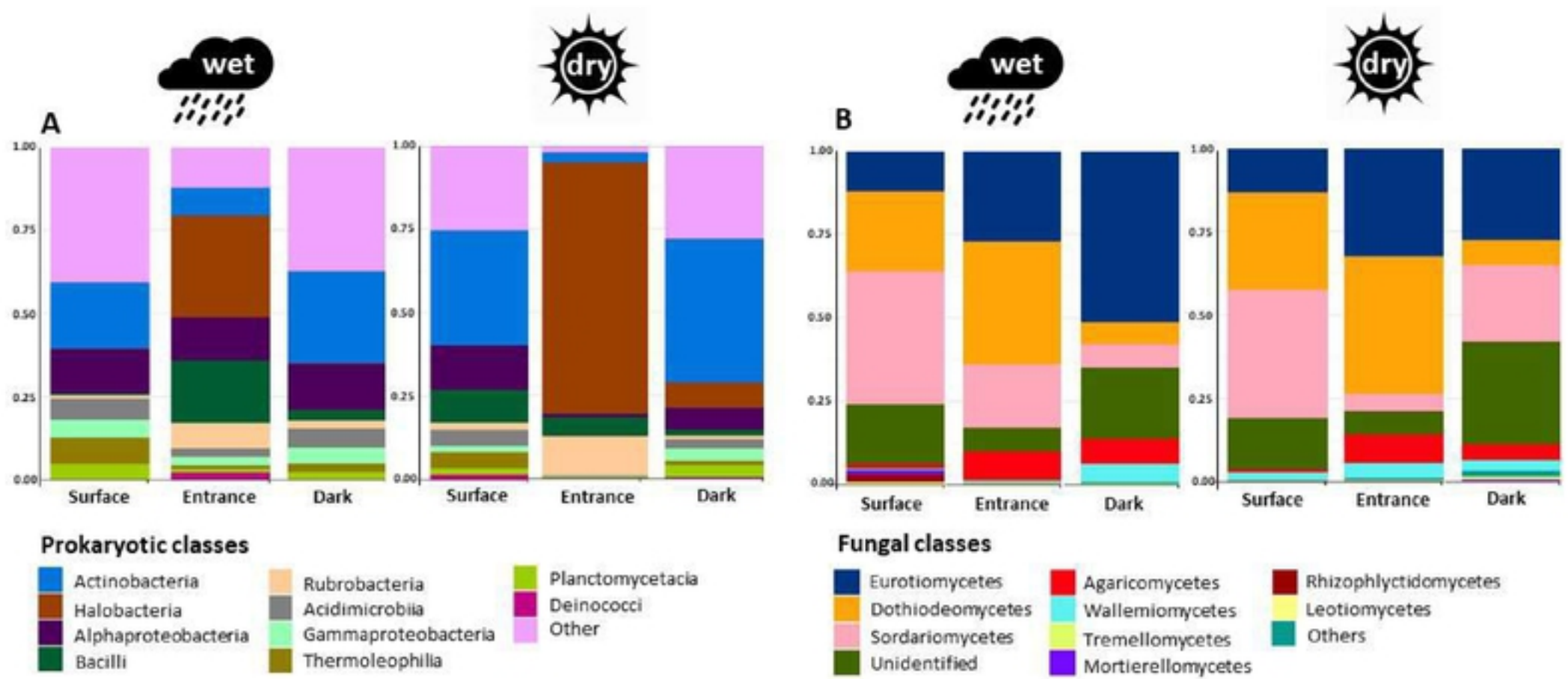
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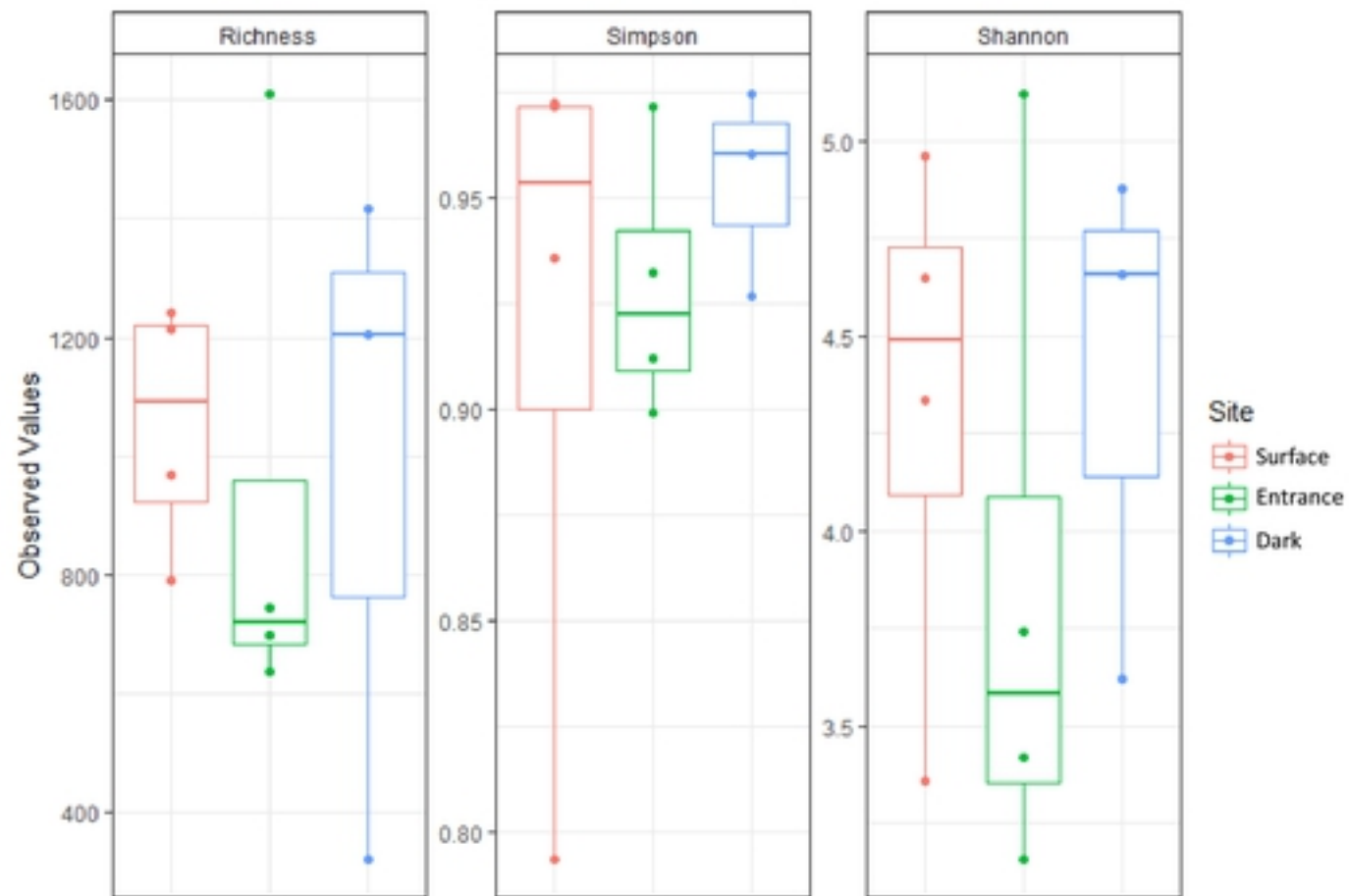
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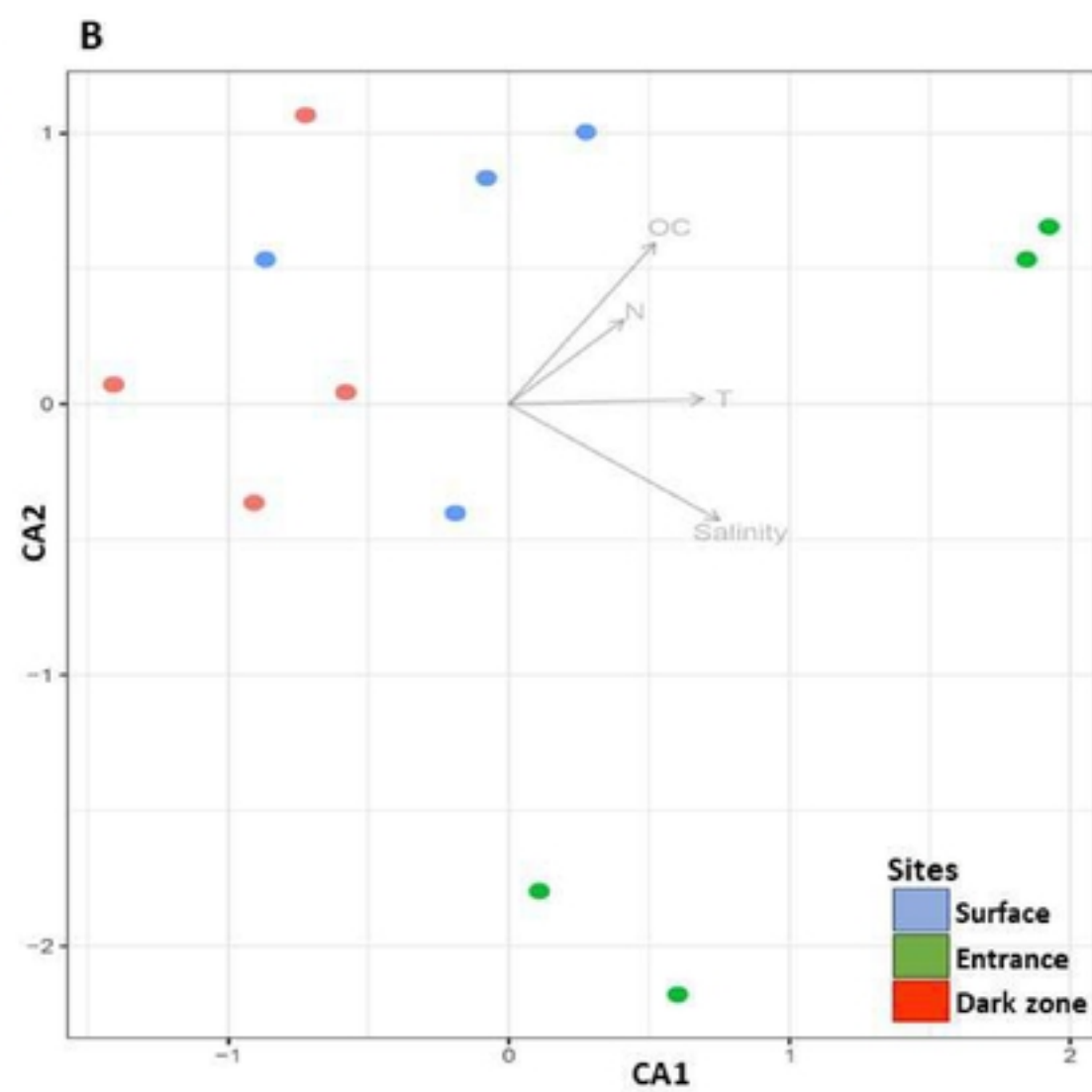
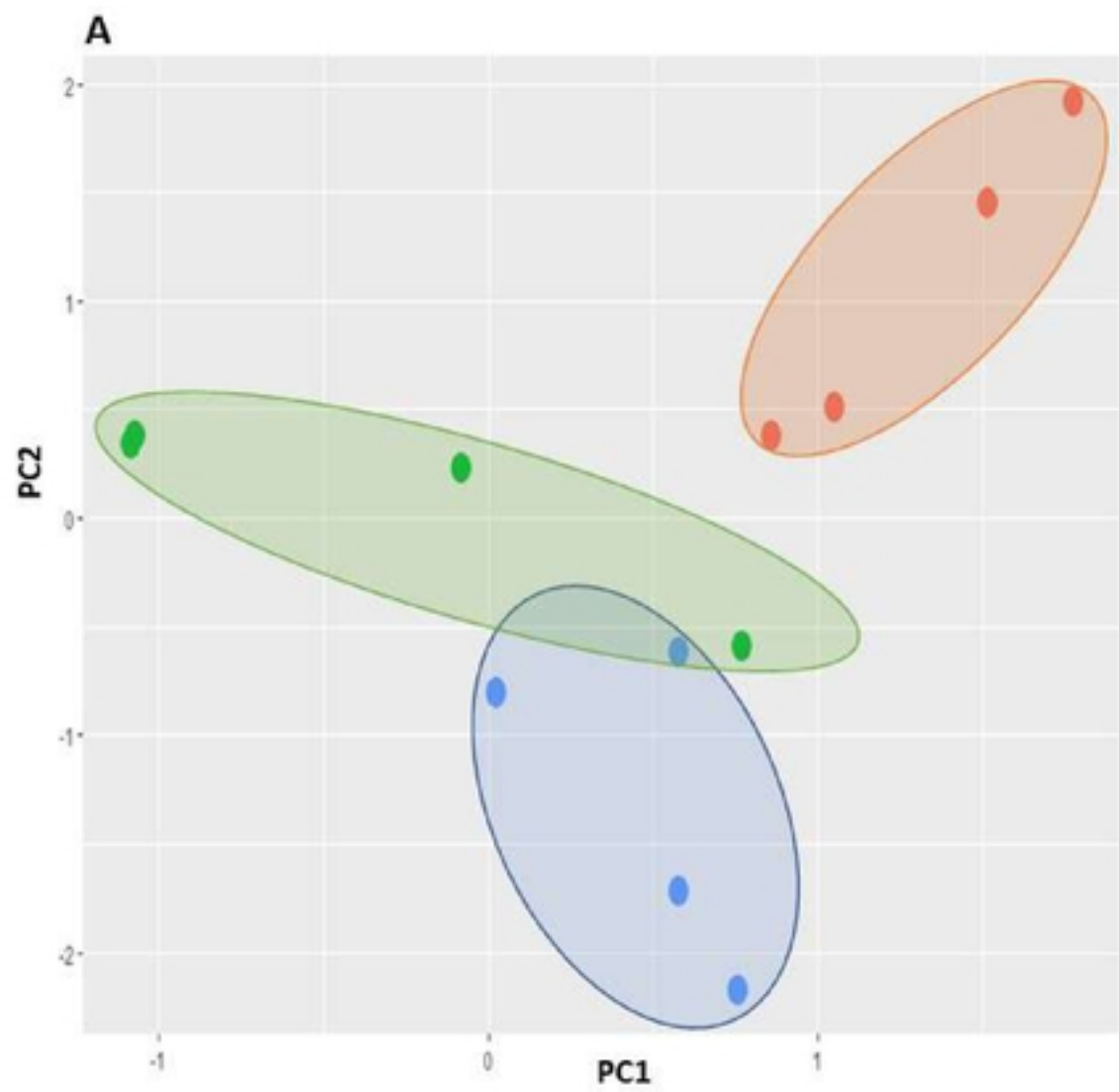
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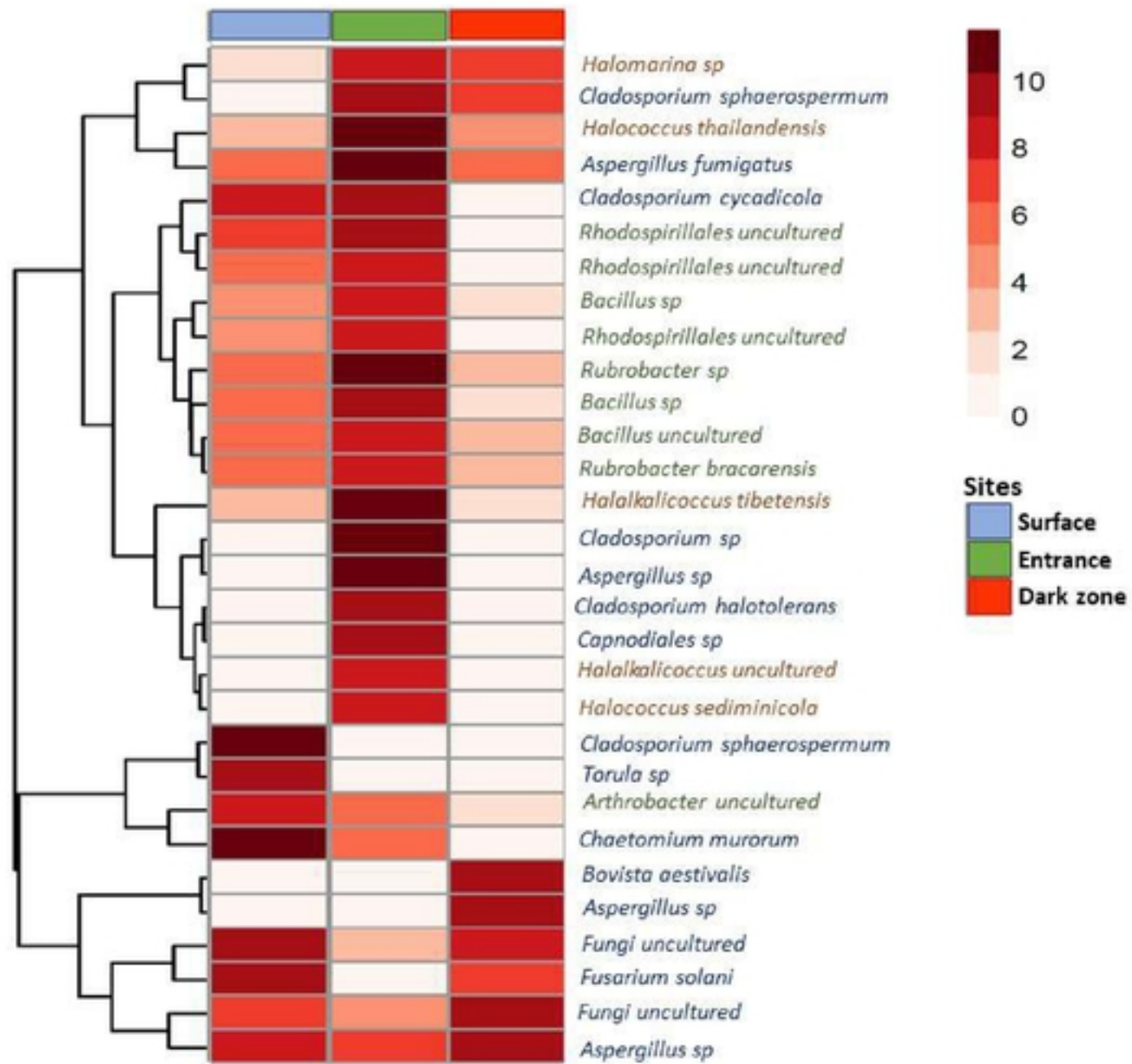
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