Diverse methanogens, bacteria and tannase genes in the feces of the endangered volcano rabbit (*Romerolagus diazi)*

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Background. The volcano rabbit is the smallest lagomorph in Mexico, it is monotypic and endemic to the Trans-Mexican Volcanic Belt. It is classified as endangered by Mexican legislation and as critically endangered by the IUCN, in the Red List. *Romerolagus diazi* consumes large amounts of grasses, seedlings, shrubs, and trees. Pines and oaks contain tannins that can be toxic to the organisms which consume them. The volcano rabbit microbiota may be rich in bacteria capable of degrading fiber and phenolic compounds. **Methods.** We obtained the fecal microbiome of three adults and one young rabbit collected in Coajomulco, Morelos, Mexico. Taxonomic assignments and gene annotation revealed the possible roles of different bacteria in the rabbit gut. We searched for sequences encoding tannase enzymes and enzymes associated with digestion of plant fibers such as cellulose and hemicellulose. **Results.** The most representative phyla within the Bacteria domain were: Proteobacteria, Firmicutes and Actinobacteria for the young rabbit sample (S1) and adult rabbit sample (S2), which was the only sample not confirmed by sequencing to correspond to the volcano rabbit. Firmicutes, Actinobacteria and Cyanobacteria were found in adult rabbit samples S3 and S4. The most abundant phylum within the Archaea domain was Euryarchaeota. The most abundant genera of the Bacteria domain were *Lachnoclostridium* (Firmicutes) and *Acinetobacter* (Proteobacteria), while *Methanosarcina* predominated from the Archaea. In addition, we obtained 18 bacterial tannase sequences. The potential functions of these sequences were identified which include carbohydrate and amino acid metabolism. We also found genes encoding enzymes for plant fiber degradation such as β-1-4-endoglucanases, xylanases, β-glucosidases and arabinofuranosidases.

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Abstract

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32 consumes large amounts of grasses, seedlings, shrubs, and trees. Pines and oaks contain tannins

33 that can be toxic to the organisms which consume them. The volcano rabbit microbiota may be

34 rich in bacteria capable of degrading fiber and phenolic compounds.

35 **Methods.** We obtained the fecal microbiome of three adults and one young rabbit collected in

36 Coajomulco, Morelos, Mexico. Taxonomic assignments and gene annotation revealed the

37 possible roles of different bacteria in the rabbit gut. We searched for sequences encoding tannase

38 enzymes and enzymes associated with digestion of plant fibers such as cellulose and

39 hemicellulose.

40 **Results.** The most representative phyla within the Bacteria domain were: Proteobacteria,

41 Firmicutes and Actinobacteria for the young rabbit sample (S1) and adult rabbit sample (S2),

42 which was the only sample not confirmed by sequencing to correspond to the volcano rabbit.

43 Firmicutes, Actinobacteria and Cyanobacteria were found in adult rabbit samples S3 and S4. The

44 most abundant phylum within the Archaea domain was Euryarchaeota. The most abundant

45 genera of the Bacteria domain were *Lachnoclostridium* (Firmicutes) and *Acinetobacter*

46 (Proteobacteria), while *Methanosarcina* predominated from the Archaea. In addition, we

47 obtained 18 bacterial tannase sequences. The potential functions of these sequences were

48 identified which include carbohydrate and amino acid metabolism. We also found genes

49 encoding enzymes for plant fiber degradation such as β-1-4-endoglucanases, xylanases, β-

50 glucosidases and arabinofuranosidases.

51

52 **Key words:** microbiome, fecal metagenome, herbivorous animals, tannase enzymes, volcano

53 rabbit

54 **Introduction**

55   Mammals establish symbiotic relationships with microorganisms that colonize different

56   regions of the body, such as skin, mucous membranes, gastrointestinal tract, among others

57   (Muegge et al. 2011). Microbiota is defined as a microbial community living in an anatomical

58   site, self-regulating their abundance and metabolic dynamics, which can influence the health

59   status of the host. Microbiome is the term used to designate the genome of the microbiota

60   (Redinbo 2014; Ventura et al. 2009). Herbivores have been characterized as having a larger

61   microbial diversity in their guts than omnivores or carnivores (Ley et al. 2008). Bacterial

62   symbionts expand the host digestion spectrum by fermenting cellulose and hemicellulose (Ley et

63   al. 2008; Tilg 2010). Individual/intrinsic factors that includes age, sex, genetics, host phylogeny

64   and environmental/extrinsic factors like diet and habitat conditions could change the microbial

65   community of the rabbit gut (Funosas et al. 2021; North et al. 2019).

66   The rabbit gastrointestinal tract presents a 6-6.5 pH, associated with high humidity (75-

67   95%) and stable temperature of 35-40 ºC. The transit speed of particles and food fluids is slower

68   (27 and 39 hours respectively) compared to other species as the guinea pig (13 hours), rat (22

69   and 20 hours) and horse (25 and 20 hours) (Velasco-Galilea et al. 2018). The rabbit gut microbial

70   community is composed of 100-1000 billion microorganisms per gram of cecal, belonging to

71   1000 different species of microorganisms (Combes et al. 2011). Bacteria belonging to

72   Lachnospiraceae, Clostridiaceae, and Ruminococcaceae families play an important role in the

73   digestion of cellulose and hemicellulose as they may produce short-chain fatty acids (Biddle et

74   al. 2013). In addition, other families that include Desulfovibrionaceae, Eubacteriaceae,

75   Bacteroidaceae, Christensenellaceae, Erysipelotrichaceaea, Rikenellaceae and Spirochaetaceae

76   have been reported in lagomorphs (North et al. 2019; Shanmuganandam et al. 2020; Stalder et al.

77   2019).

78   Diet is considered one of the factors modeling the microbial community in all animals

79   and this is also the case with hindgut-fermenter animals like rabbits (Muegge et al. 2011).

80   Moreover, rabbits are caecotrophagic animals (they ingest their own soft fecal pellets produced

81   by digestion in the cecum) profiting from the nutrients in feces derived from microbial

82   fermentation (Crowley et al. 2017). Fecal ingestion would allow a second digestion of plant

83   fibers, a process that could be considered analogous to ruminant digestion. Coprophagy is as well

84   a very efficient way to recycle gut microbiota (Martinez-Romero et al. 2020). In most lagomorph

85   studies, Firmicutes were found as the most abundant bacteria (North et al. 2019; Stalder et al.

86   2019; Velasco-Galilea et al. 2018) followed by Bacteroidetes (North et al. 2019; Stalder et al.

87   2019), although Crowley et al. (2017) found that both Bacteroides and Firmicutes were equally

88   abundant. Proteobacteria were found to be more abundant in cecal samples (North et al. 2019),

89   which differs from the results with fecal samples investigated in other papers (Velasco-Galilea et

90   al. 2018; Shanmuganandam et al. 2020). It should be noted that Illumina 16S v3-v4 sequencing

91   led to an underrepresentation of Firmicutes (Shanmuganandam et al. 2020). Firmicutes and

92   Bacteroidetes are the dominant phyla in human guts (Human Microbiome Project 2012) and

93   therein the Firmicutes/Bacteroidetes ratio is not constant and changes with age (Mariat et al.

94   2009) and in some cases with obesity (Magne et al. 2020).

95   The volcano rabbit (*Romerolagus diazi*) is an endemic species classified as endangered

96   by Mexican legislation (SEMARNAT 2010) and as critically endangered in the IUCN Red List

97   (Velázquez & Guerrero 2019). It plays an important ecological role as part of the diet of

98   carnivorous mammals, prey for birds and reptiles (Cervantes & Martínez-Vázquez 1996;

99   Uriostegui Velarde et al. 2015). Besides, the volcano rabbit consumes large amounts of

100   vegetative parts of grasses, seedlings, shrubs, trees and can regulate plant composition and seed

101 dispersal processes (Granados-Sánchez et al. 2004). The diet of the volcano rabbit is based on

102 grasses but is complemented with other plants. Previously, 37 plant species were identified in its

103 diet, 80% of which were grasses such as *Muhlenbergia macroura*, *Festuca amplissima* and

104 *Jarava ichu*. In addition, the consumption of leaves of *Phoradendron velutinum*, *Pinus* sp, and

105 *Quercus laurina* was recorded (Mancinez Arellano 2017). Pines and oaks contain chemical

106 compounds that can be toxic to the organisms that consume them. These toxins are considered to

107 have a defensive role against animals (Granados-Sánchez et al. 2008). The aim of this study was

108 to describe the microbes found in the volcano rabbit feces to assess their role in plant fiber and

109 tannin degradation, because tannins are important constituents of their diet. We surmised that the

110 fecal microbiome of the volcano rabbit would reflect its herbivorous diet as observed in other

111 animals (Martinez-Romero et al. 2020) with a plethora of genes encoding enzymes to degrade

112 plant fibers and phenolics.

113

114 **Materials & Methods**

115 **Sample collection**

116   Four fecal samples were collected from Coajomulco, Morelos Mexico (14Q UTM

117 478960.07 m E, 2109691.32 m N) under a permit by Secretaría de Medio Ambiente y Recursos

118 Naturales (SGPA/DGVS/006985/18). These pellets are ochre in color, with a smooth, shiny

119 texture and their maximum diameter is one centimeter in adults. We kept more than 25 m

120 between one latrine and another within a collection site, this distance is the estimated home range

121 for these rabbits (Cervantes & Martínez-Vázquez 1996), except for two samples (S1 and S2) that

122 were collected in the same latrine with 0.1 m distance between them, but the pellets were of

123   different sizes. Feces were stored individually in Eppendorf-type tubes in an icebox and later

124   taken to the laboratory and stored in a refrigerator at 4 °C for 6 hours.

125   **DNA extraction, sequencing, and Illumina library generation**

126   Total DNA of the four samples was extracted using the commercial DNA Isolation Kit

127   from Roche Life Science. A total of 230 mg of feces were processed for each sample. DNA

128   concentration was measured by Nanodrop (Thermo Scientific) and Qubit™ dsDNA HS Assay

129   Kits) and the quality was determined by visualization in a 1% agarose gel. Samples requirements

130   for the preparation of the libraries were: DNA concentration > 200 ng/μl, total amount of DNA >

131   1 μg and DNA bands visualized in agarose had to be clear and of good quality. The genomic

132   DNA was randomly cut into small fragments and subcloned into a "universal" cloning vector.

133   The sub fragment library was randomly sampled, and several sequences reads (using a universal

134   primer that directs sequencing from within the vector) were generated from both strands.

135   Sequencing was obtained with Illumina HiSeq 4000 using paired-end 2 × 151 bp, at the

136   sequencing unit Functional Genomics Laboratory at UC Berkeley, California.

137   **Sequence filtering and metagenome assembly**

138   Raw fastq reads were quality filtered with FASTP 0.20.0 (Chen et al. 2018). We

139   considered good quality reads when the quality score was equal to or greater than 30. Clean

140   reads were mapped against genomes such as human, rat, rabbit, yeast, mouse, pine, oak, fly,

141   worm, adapters, and pika to know whether the reads had contamination using FastQ Screen

142   0.14.0 (Wingett & Andrews 2018). Data is available in the SRA at NCBI, BioProject:

143   PRJNA721235, Accession number: SRR14209496, SRR14209495, SRR14209494,

144   SRR14209493). Metagenome assembly was performed using metaSPADES 3.12.0 (Nurk et al.

145   2017) and MEGAHIT 1.1.3 (Li et al. 2015). Comparison of the assemblies was conducted to

146   identify the best assembly with MetaQUAST 5.0.2 (Mikheenko et al. 2016). A good quality

147   assembly considers the proportion of contigs < 1000nt, total length of the contigs, longest contig,

148   N50 (length), L50 (position), fewer contigs and identifying single-copy genes (> 90%) (Nurk et

149   al. 2013). Clean reads (R1 and R2) were aligned to each corresponding assembly to obtain the

150   percentage of reads that were assembled using Bowtie2 2.4.2 (Langmead & Salzberg 2012). We

151   confirmed that the samples were from volcano rabbits because we encountered DNA sequences

152   in feces that matched those reported from the same rabbit species using the nucleotide sequences

153   of the cytochrome *b* gene. We assessed taxonomic identity on fecal samples using Blast 2.10.0+

154   (Camacho et al. 2009) and a phylogenetic reconstruction using maximum likelihood (ML).

155   Additional sequences of related organisms were obtained by Blast 2.10.0+ at NCBI. Sequences

156   were aligned with Mafft 7.149b (Katoh & Standley 2013). The best substitution model

157   (TPM2+F+G4) and ML analysis were performed with IQ-TREE 1.6.12 (Trifinopoulos et al.

158   2016).

159   **Taxonomic Assignment**

160   The microbial taxonomy assignation of each assembly per sample was estimated using

161   Kraken 2 2.0.8 (Wood et al. 2019). The abundance of the microbial community by phylum and

162   genus was estimated using Bracken 2.5.0 (Bayesian Abundance Re-estimation with KrakEN).

163   Bracken could reassign sequences that kraken 2 could not classify within the genus or species

164   levels with a reliable percentage above 98% (Lu et al. 2017). Both programs were run against a

165   Minikraken2_v1 database containing bacterial, archaeal genomes and viral libraries. In addition,

166   we searched for the 16S rRNA gene sequences in the metagenomes by Blast 2.10.0+. Redundant

167   sequences were removed with CD-HIT 4.8.1 (Li & Godzik 2006). Phylogenetic reconstruction

168   was performed using the maximum likelihood (ML) method. Additional sequences of related

169   organisms were obtained by Blast at NCBI and with the Refseq-RDP database. Sequences were

170   aligned with Mafft 7.149b. Spurious bases, editing and trimming were performed with trimAl 1.4

171   (Capella-Gutierrez et al. 2009). The best substitution model (GTR+F+R5) and ML analysis were

172   performed with IQ-TREE 1.6.12.

173   **Alpha diversity and microbial composition**

174   The microbial diversity of each sample at both phylum and genus level of Bacteria and

175   Archaea was estimated with the Hill numbers in terms of effective numbers of elements under

176   the same sample coverage (Chao & Jost 2012). Hill numbers are a mathematically unified family

177   of diversity indices differing only by an exponent q (^{q}D) (Chiu & Chao 2016), where $q = 0$ is

178   equivalent to species richness, $q = 1$ corresponds to the exponential of Shannon entropy

179   (effective number of common elements), and $q = 2$ is equivalent to the inverse of Simpson index,

180   interpreted as the effective number of dominant elements (Ma & Li 2018; Ma et al. 2019). To

181   compare the microbial diversity between the four samples, we used the 95% confidence intervals

182   (CI), where no overlap between CI values indicates significant differences (Cumming et al.

183   2007). The qD diversity, sample coverage, and their respective confidence intervals were

184   obtained with the iNEXT R package 3.5.3 (Hsieh et al. 2016), using as endpoint the maximum

185   number of contigs in each sample and 1,000 bootstraps for the construction of the rarefaction

186   curves and CI.

187   The difference in composition of bacterial and archaeal communities was assessed by

188   one-way ANOSIM based on Bray-Curtis similarity metrics and with 10,000 permutations. When

189   the ANOSIM was significant, a pairwise comparison was conducted between samples using the

190   Bonferroni p-value correction as implemented in Past 4.2 (Hammer et al. 2001). This analysis

191   was conducted considering only those genera with relative abundance equal to or greater than

192   0.5%, meaning that rarely occurring microorganisms were not considered.

193   **Predicted functional annotation**

194   Gene annotation and coding sequence identification in feces microbiomes were

195   performed using Prokka 1.12 (Seemann 2014). One step for cleaning the protein fasta file-to

196   eliminate redundant sequences was performed using CD-HIT. We used blastp 2.10.0+ on the

197   sequences that Prokka did not score (hypothetical proteins) against the Uniprot-Sprot database.

198   Additionally, non-redundant protein fasta files of each sample were obtained. We performed a

199   second annotation of protein sequences with the online program GhostKOALA 2.2 (Kanehisa et

200   al. 2016), using GHOSTX search (it uses suffix arrays to find matching sequences and runs 100

201   times faster than BLAST), against a non-redundant set of KEGG genes (Kyoto Encyclopedia of

202   Genes and Genomes).

203   # Results

204   **Sequence and metagenome assembly**

205   We extracted DNA from four fecal samples. According to the size of the collected feces,

206   S1 (0.5 cm) corresponded to a young individual, S2, S3 and S4 (1.0 cm) corresponded to adult

207   rabbits (Velázquez et al. 1996). We confirmed that the feces samples 1, 3, and 4 were from

208   volcano rabbits because we found cytochrome *b* sequences in feces matched those reported

209   previously from the same rabbit species (Osuna et al. 2020). Although cytochrome *b* sequence

210   was not detected from sample S2, we are confident that it corresponds to volcano rabbit as feces

211   of other rabbits inhabiting the zone are clearly distinctive (Velázquez et al. 1996). Maximum

212   likelihood analysis indicated that the cytochrome *b* gene sequences found in the metagenomes

213   were phylogenetically placed with the volcano rabbit (Fig. S1).

214   We obtained a total of 332.8 million raw reads from the four samples. Once the cleaning

215   was completed, we rescued 316.6 million reads and the average number of reads per sample was

216   79.15 million (ranging from 69.6 to 96.6) (Table S1). Extra filtering of the clean sequences was

217   conducted to obtain external contamination. The results demonstrated that between 98.2% and

218   99.6% of the clean reads were not assigned to adapters, human, rat, rabbit, yeast, mouse, pine,

219   oak, fly, worm, and pika genomes. Individual assemblies of each sample S1, S2, S3 and S4 were

220   performed. Fewer and larger contigs were obtained with metaSPADES than with MEGAHIT.

221   Moreover, 83% – 85 % of the clean reads were mapped against each assembly.

222   **Taxonomic assignment**

223   The percentage of contigs that could be classified ranged between 14% and 18% for the

224   taxonomic levels of phylum and genus of the Bacteria and Archaea domains. This could be due

225   to the limitations of the non-human database where rare species are present (Tamames et al.

226   2019). Twenty-nine phyla were obtained from the Bacteria domain (Fig. 1a). The phylum

227   Proteobacteria was the most abundant in sample two (S2) and sample from the young rabbit (S1)

228   that has been found in other young animals and humans (Moon et al. 2018). The most abundant

229   phylum in samples S3 and S4 was Firmicutes. Additionally, we found three phyla of the Archaea

230   domain: Euryarchaeota, Crenarchaeota and Thaumarchaeota with the most abundant being

231   Euryarchaeota (Fig. 1b). Nine families were the most abundant in all samples (Table 1). The

232   most abundant genus of the domain Bacteria in the young rabbit feces was *Acinetobacter*

233   (Proteobacteria) while in adult-rabbit feces was *Lachnoclostridium* (Firmicutes) (Fig. 2a).

234   *Candidatus tachikawaea*, *Leclercia*, *Candidatus riesia*, and *Obesumbacterium* were only found

235   in the gut metagenome from young rabbit feces (Fig. 2a). The number of archaeal genera we

236   encountered here in the fecal microbiome of the volcano rabbit is remarkable. We identified 15

237   genera of the domain Archaea, *Methanosarcina*, *Methanoculleus* and *Methanococcus* were the

238   most abundant in all samples. The genus *Halorabdus* was the most abundant only in sample two

239   (Fig. 2b), which is the only sample for which there was no confirmation of host rabbit identity.

240   We recovered 169 16S rRNA sequences (1273 ~ 1500 bp) from the assembled

241   metagenomes. Maximum likelihood analysis indicated that the 16S rRNA gene sequences found

242   in the metagenomes were phylogenetically placed in the following phyla: Firmicutes,

243   Bacteroidetes, Proteobacteria, Synergistetes, Tenericutes, Verrucomicrobia, Cyanobacteria,

244   Lentisphaerae and Spirochaetes (Fig. S2). Besides, we found some 16S rRNA gene sequences

245   that did not cluster with any reported phyla. Many 16S rRNA gene sequences were related to the

246   species *Marvinbryantia formatexigens*, which has been found in the human gut and has been

247   reported to degrade plant oligosaccharides (stachyose and raffinose) (Rey et al. 2010).

248   **Alpha diversity and microbial composition**

249   In all samples, at the phylum and genus level of Bacteria and Archaea showed a sample

250   coverage of 100%, indicating that sampling is complete for all samples (Fig. 3). Then the

251   diversity comparisons were made directly according to their confidence intervals (Chao & Jost

252   2012). For bacterial communities, the richness of S1 was significantly higher than in the other

253   samples, at both phylum (Fig. 4a) and genus level (Fig. 4c). For the phyla of Archaea domain,

254   the richness of both S1 and S2 were not significantly different while a similar pattern was

255   observed between S3 and S4 (Fig. 4b). Richness estimates at the genus level in both domains

256   differed significantly between all samples (Fig. 4c; Fig. 4d). The diversity values of the effective

257   number of common and dominant elements are shown in Table S2.

258   According to ANOSIM, community composition at Bacteria and Archaea domains was

259   not significantly different between samples ($p > 0.05$). For genera of domain Bacteria, the

260 composition was significantly different between S1 and the other three samples (p = 0.0002),

261 while for the genera of domain Archaea the composition was significantly different between all

262 samples (p = 0.0002).

263 **Functional annotation**

264   A similar number of sequences was annotated from all samples (101,832 - 138,018) in all

265 categories (Table 2). According to the function of their specific catalytic action, genes encoded

266 oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases (Chang et

267 al. 2021). The most abundant genes in all samples were transferases (28,523 - 21,324 sequences)

268 and hydrolases (17,885 - 24,675) followed by oxidoreductases (10,797 - 7,757), ligases (5,081-

269 7,013), lyases (4,896 - 6,064) and isomerases (4,227-5,500), no translocase-type enzymes were

270 found with the PROKKA annotation (Fig. 5). The highest number of annotations of all the

271 enzymes (82,572) was in S1 while the sample with the lowest number of annotated enzymes was

272 S2 (61,201) (Fig. 5). Additional enzyme search using blastp showed between 954 - 1,125 total in

273 different samples. Genes encoding translocases were recovered, which ranged from 16 to 37

274 sequences in the different samples. We found 21 functional categories. Protein Families (17,065

275 - 11,711 contigs per sample), genetic information processing (17,065 - 12,536), carbohydrate

276 metabolism (glycolysis/gluconeogenesis, citrate cycle, TCA cycle; 12,571 - 9,776), protein

277 families: signaling and cellular processes (7,400 - 5,197) and amino acid metabolism (7,103 -

278 5,730) were the most abundant in all samples. Meanwhile, xenobiotics biodegradation (127 - 58)

279 and biosynthesis of secondary metabolites (49 - 66) had the lowest number of annotated

280 sequences (Fig. 6).

281   Tannase enzyme sequences from different taxonomic groups were searched and

282 downloaded from Uniprot and NCBI databases. Blastp search was performed to obtain the

283 possible gene sequences for tannase enzymes. 18 sequences were found in the four metagenomic

284 samples of volcano rabbits that were affiliated to phylum Verrucomicrobia, family

285 Ruminococcaceae, *Victrivallis vadensis*, *Blautia* sp. and *Clostridium* sp. Finally, enzymes that

286 participate in the degradation of cellulose and hemicellulose of grasses were searched. The

287 highest number of sequences was obtained from young rabbit feces (849), while the lowest

288 number was obtained in S2 (526) (Fig. 7). The most abundant were endo 1,4 β-xylanases for

289 samples S1 (363) and S4 (248) and arabinofuranosidases for samples S2 (160) and S3 (149). The

290 enzymes found in lower proportion were those encoding β-glucosidases in sample S2 (94) and

291 Endo-glucanases in samples S3 (142) (Fig. 7).

Discussion

Gut microbiota

294   There are few studies on the microbiota of wild Lagomorphs. To our knowledge, this is

295 the first one to characterize the fecal microbiome of the volcano rabbit (*R. diazi*) that is endemic

296 to central highlands in Mexico. As an herbivore, the volcano rabbit would feed on plants that

297 contain endophytes which may become part of the gut microbiota. This occurs in other

298 herbivorous animals' endophytes that have tannases and enzymes to degrade plant cell wall

299 components (Martinez-Romero et al 2020). A critical issue that should be considered is whether

300 the microbiota identified from herbivorous animals represent *bona fide* gut bacteria or reflect

301 endophytes that are still contained within the plant tissue fragments macerated during DNA

302 extraction. Here a mild procedure not including physical maceration was performed and all plant

303 debris was eliminated by centrifugation.

304   Diet changes may have fast and important consequences in gut microbiota (Human

305 Microbiome Project 2012). The uncontrolled diets of *R. diazi* in their natural habitat may explain

306   differences among adult samples. Otherwise, differences could be due to the distinct lapse from

307   fecal excretion and collection for which we have no record. Peculiarities of the gut microbiota of

308   babies, infants and young animals have been recurrently reported, and here we corroborated this

309   with the only young rabbit that was sampled. There are reports from three-week-old Asian

310   elephants (*Loxodonta cyclotis*) (Ilmberger et al. 2014), young bats (*Leptonycteris yerbabuenae*)

311   (Gaona et al. 2019) and puppies and kittens (Moon et al. 2018) where Proteobacteria are

312   abundant. Since the gut of newborns is rich in oxygen, the role of Proteobacteria may be

313   involved in oxygen consumption, thus preparing the neonatal gut for colonization by strict

314   anaerobic microorganisms necessary for a healthy gut (Shin et al. 2015).

315   Here we report that the fecal microbiome of the volcano rabbit was dominated by

316   Proteobacteria, Firmicutes, Actinobacteria, for S1 and S2 and Firmicutes, Actinobacteria and

317   Cyanobacteria for S3 and S4. We suggest that the similarity between S1 and S2 may be due to

318   the individuals being related because young rabbits tend to be close to their mothers and these

319   samples were collected at the same location. However, we remark that in zoos and in houses,

320   rabbits would have an artificial diet. It is also worth noting that fecal and gut bacteria are not the

321   same and more Proteobacteria were found in intestinal samples than in fecal samples in the

322   European brown hare (Stalder et al. 2019). Additionally, Proteobacteria has been reported to be

323   more abundant in rabbits with the symptoms of epizootic rabbit enteropathy (ERE) (Bauerl et al.

324   2014) and in other mammals, such as bats (*Artibeus lituratus*) (Ingala et al. 2018) and Asian

325   elephants with a high fruit diet (Budd et al. 2020). On the other hand, a study in New Zealand

326   white rabbits has reported a high abundance of-Actinobacteria and Tenericutes (North et al.

327   2019). Phylum Lentisphaerae was previously reported in hares (*L. europaeus*).

328   On the other hand, 16S rRNA reads from shotgun metagenomics provide sequences that

329   are not subject to PCR primer bias and covers taxa that are not detected by primer sets (Yuan et

330   al. 2015); therefore, we complemented the kraken 2 taxonomic assignment with a global 16S

331   rRNA gene bacterial identification and phylogeny from metagenomes. We obtained nine phyla

332   out of 29 in the 16S rRNA analysis. Phylum Firmicutes was the most abundant in both. It has

333   been reported that kraken 2 is suitable for the classification of gut microbiome with assemblies'

334   recruit of 85% or more of the original raw reads (Tamames et al. 2019). Nevertheless, samples

335   that have more diversity and complexity would not be correctly classified with kraken 2. Less

336   studied habitat metagenomes such as non-human environments show fewer similarities with

337   kraken 2 which is quite sensitive to the composition of databases and their performance

338   decreases when rare species are present in the metagenome (Tamames et al. 2019).

339   Domain Archaea has been reported in Caldes rabbit microbiome (Velasco-Galilea et al.

340   2018) with a single genus *Methanobrevibacter* (Euryarchaeota). Similarly, genus

341   *Methanobrevibacter* was identified in a molecular profiling from a rabbit caecum (Kusar, 2010).

342   These differences could be due to different strategies to characterize microbiota (16S amplicon

343   sequencing vs shotgun). Here we found an unusually high number (15) of archaeal genera in the

344   fecal microbiome of the volcano rabbit. Surprisingly, the most abundant genera were

345   *Methanosarcina*, *Methanoculleus*, *Methanococcus*, *Halorhabdus* (Euryarchaeota) besides

346   *Methanobrevibacter*. All of them have been found in the human gut microbiome (Borrel et al.

347   2020). *Halorhabdus* is usually associated with microaerophilic and halophilic environments. The

348   axenic species described may use the monosaccharides glucose, fructose, and xylose for growth

349   (Waino et al. 2000). Xylose is produced mainly in plants (wood, fibers, fruits) and as a

350   degradation product of hemicellulose. Except for *Halorhabdus*, the other archaea may perform

351   the terminal step in the degradation of organic matter and produce energy and methane through

352   methanogenesis pathways (Ferrer & Pérez 2010; Ferry 2020). These methanogenic archaea are

353   generally associated with strict anaerobic environments, due to the high sensitivity to oxygen

354   exposure of the enzymes involved in methanogenesis and to the lack of genes that code for

355   proteins involved in protection against oxidative stress (Jasso-Chavez et al. 2015). Furthermore,

356   these methanogenic archaea use compounds with one or two carbons to grow, for the generation

357   of biosynthetic intermediates and polysaccharide storage (Santiago-Martinez et al. 2016).

358   *Methanoculleus*, *Methanococcus* and *Methanobrevibacter* use carbon dioxide plus hydrogen

359   (CO_2 + H_2) and formate (HCOO-), while *Methanosarcina* uses more diverse carbon sources, e.g.,

360   acetate (CH_3COO-), methanol (CH_3OH) and methylamines (CH_3NH_2), plus CO_2 + H_2 (Borrel et

361   al. 2020; Buan & Robinson 2018). These compounds are produced by the metabolism of

362   eukaryotic and bacterial species. Specifically, there is evidence that the high intake of dietary

363   fiber in animals, followed by fermentation mediated by the intestinal microbiota, changes the

364   concentration and metabolism of methylamines (Li et al. 2017), which are a very good carbon

365   source for methanogenic archaea. This suggests that the type of diet could increase the diversity

366   and abundance of methanogenic species. The diversity and abundance of methanogenic archaea

367   found in volcano rabbits may indicate a rich diversity of ingested vegetable and very complex

368   interaction between bacteria and archaea. Seemingly there is no competition for food between

369   these methanogenic and non-methanogenic archaea, due to the well-established difference in

370   preference for carbon sources.

371   **Prediction of functional annotation**

372   In the present study, we found several functional categories suggesting that the fecal

373   microbiome of *R. diazi* may have biosynthetic amino acid, carbohydrate metabolism, lipid

374 metabolism, metabolism of terpenoids, polyketides and secondary metabolites and xenobiotic

375 biodegradation capabilities. Enzymes found in the fecal microbiome of the volcano rabbit were

376 mainly transferases, hydrolases, and oxidoreductases enzymes. Categories such as carbohydrate

377 metabolism and amino acid metabolism had been reported in other herbivores (Muegge et al.

378 2011). Pathways such as bile secretion, mineral absorption, and xenobiotics biodegradation have

379 been obtained in other rabbits (Zeng et al. 2015). Recently, metabolism of terpenoids –

380 polyketides, amino acid metabolism carbohydrate, nucleotide metabolism, lipid metabolism,

381 energy metabolism, and metabolism of cofactors and vitamins pathways were reported in

382 African elephants (Budd et al. 2020).

383 Enzymatic activity of tannases was identified in phyla Firmicutes and Proteobacteria:

384 *Enterobacter*, *Weissella* and *Lactobacillus* (de las Rivas et al. 2019), *Bacillus*, *Streptococcus*,

385 *Klebsiella, Enterococcus fecalis, Pantoea agglomerans, Staphylococcus lugdunensis,*

386 *Lactobacillus plantarum*, *L. paraplantarum* (Rodríguez-Durán et al. 2010) and *Bacillus*

387 *licheniformis* (Palacio-Arango et al. 2018). In Koala feces, a Gram-negative, facultatively

388 anaerobic and tannase-producing bacterium called *Lonepinella koalarum* was isolated (Osawa et

389 al. 1995) which was not found in the microbiome of volcano rabbit feces pointing out the

390 specialization or adaptation of some bacterial species to animal hosts.

391 Fiber degrading enzymes have been reported in bacteria such as *Clostridium*,

392 *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia* and

393 *Streptomyces* (Saratale et al. 2012). *Clostridium* was abundant in S2, S3 and S4 whereas *Erwinia*

394 in S1. In all samples, we also found phylum Actinobacteria, which is abundant in soil producing

395 secondary metabolites, antibiotics and related with degrading plant cell wall components

396 (Saratale et al. 2012).

Conclusions

398   The volcano rabbit microbiome showed distinct bacterial and archaea abundances

399 compared to other lagomorphs. We found genes that encode tannases and enzymes that degrade

400 the components of the plant cell wall in the volcano rabbit microbiome. The gut microbiota may

401 contribute to the digestion of complex plant molecules. Here we found many potential functional

402 categories such as metabolism of carbohydrates and biosynthesis of amino acids and other

403 compounds as secondary metabolites. The diversity of methanogenic species could be influenced

404 by the type of diet. In addition, we observed differences between the adults and young rabbit.

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Figure 1

Phyla of the bacteria and archaea domain found in the fecal microbiome of the volcano rabbit

Relative abundance was obtained by dividing the number of contigs assigned per phylum by the total number of contigs for the Bacteria and Archaea domains, respectively. The figures were constructed considering only phyla with a relative abundance equal to or greater than 0.5%, which means that rare bacteria and archaea were not considered. A) Percentage of abundance of the main phyla from the Bacteria domain found in feces of the volcano rabbit. B) Percentage of abundance of the main phyla from the Archaea domain found in feces of the volcano rabbit

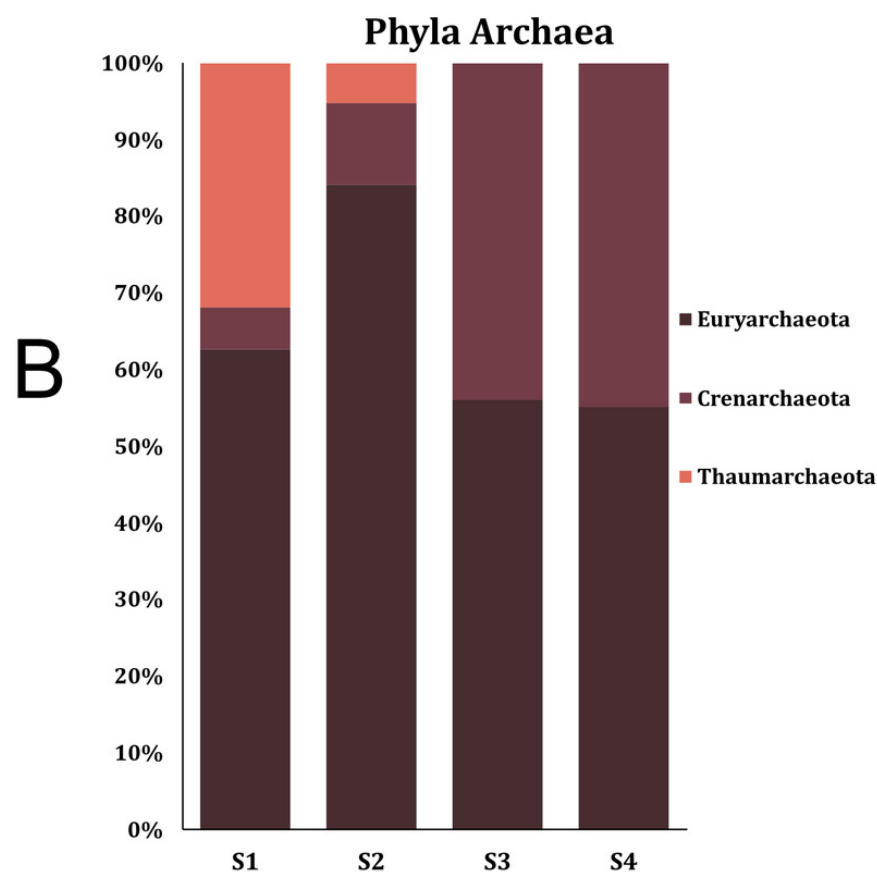
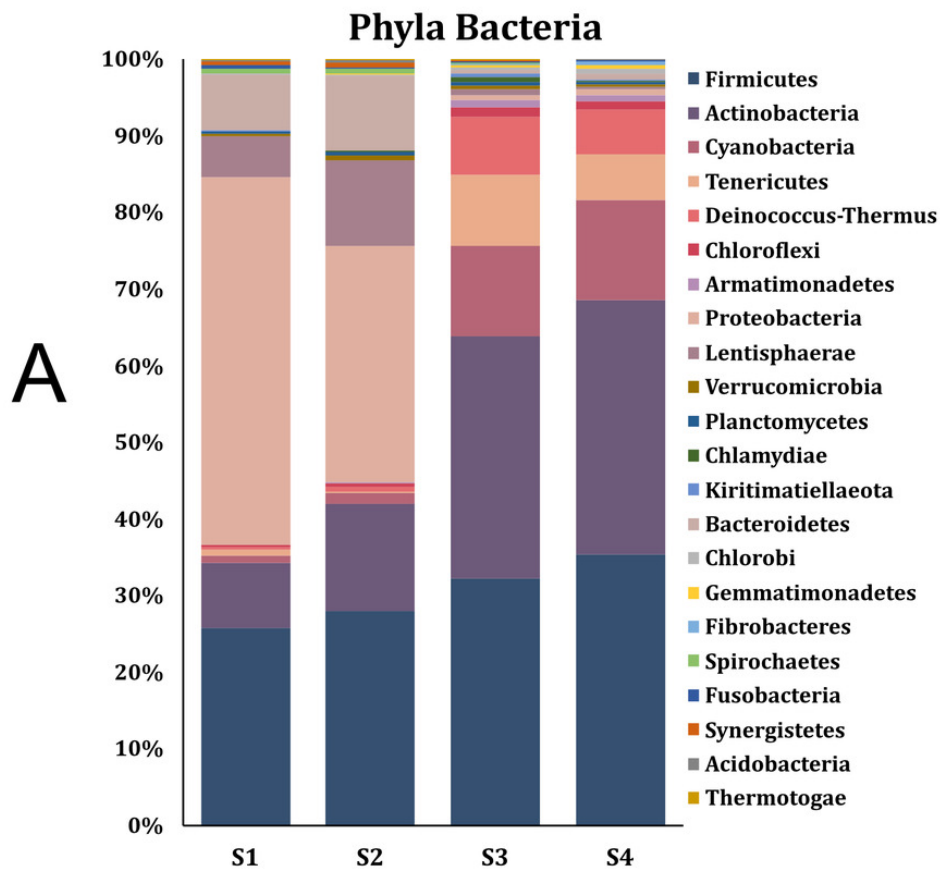



Figure 2

Genera of the bacteria and archaea domain found in the fecal microbiome of the volcano rabbit

Relative abundance was obtained by dividing the number of contigs assigned per genus by the total number of contigs for the Bacteria and Archaea domains, respectively. The figures were constructed considering only genera with a relative abundance equal to or greater than 0.5%, which means that rare bacteria and archaea were not considered. A) Percentage of abundance of the most representative genera per sample within Bacteria domain. B) Percentage of abundance of the most representative genera per sample within Archaea domain

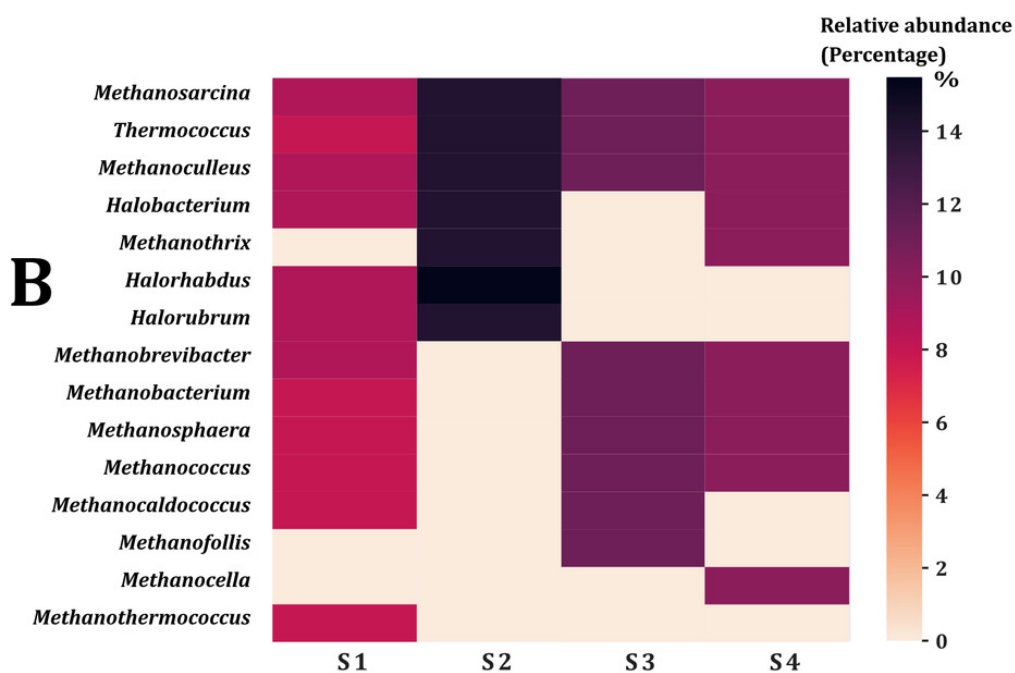
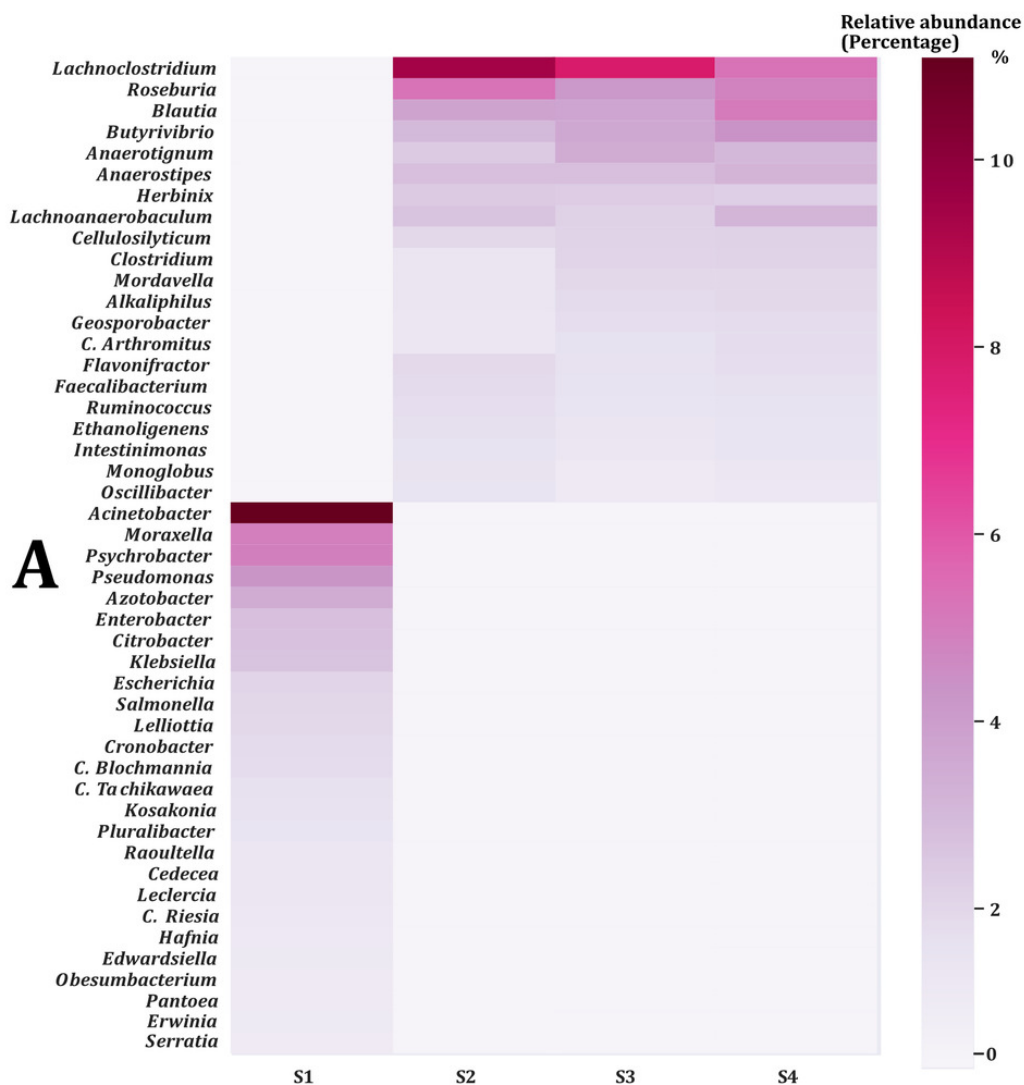



Figure 3

Sample coverage (SC) per sample, taxonomic level, and diversity (q0)

SC ranged from 0.0 to 1.0. A) Sample coverage for the phyla of the Bacteria domain. B) Sample coverage for the phyla of Archaea domain. C) Sample coverage for the genera of the Bacteria domain. D) Sample coverage for the genera of the Archaea domain

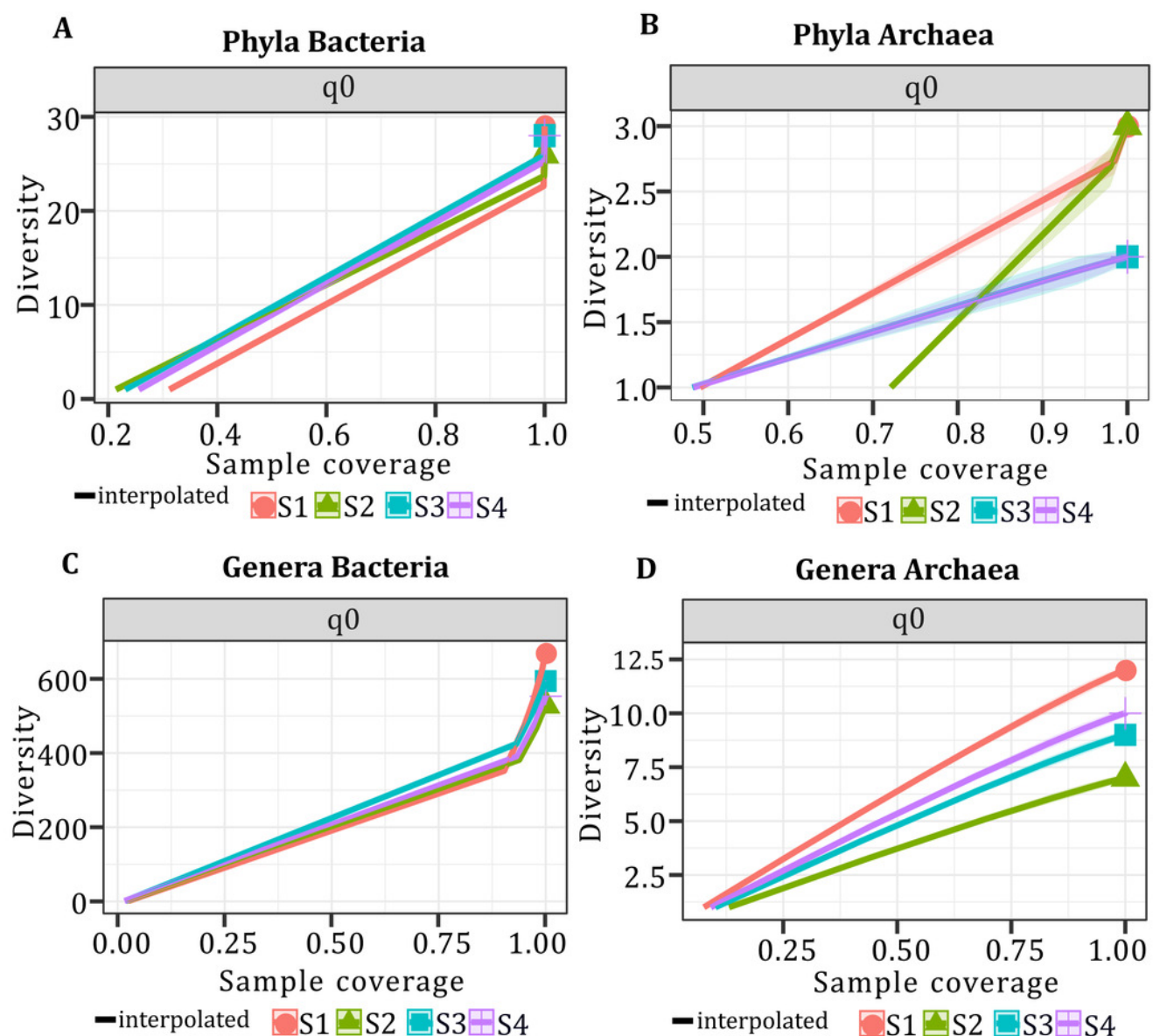



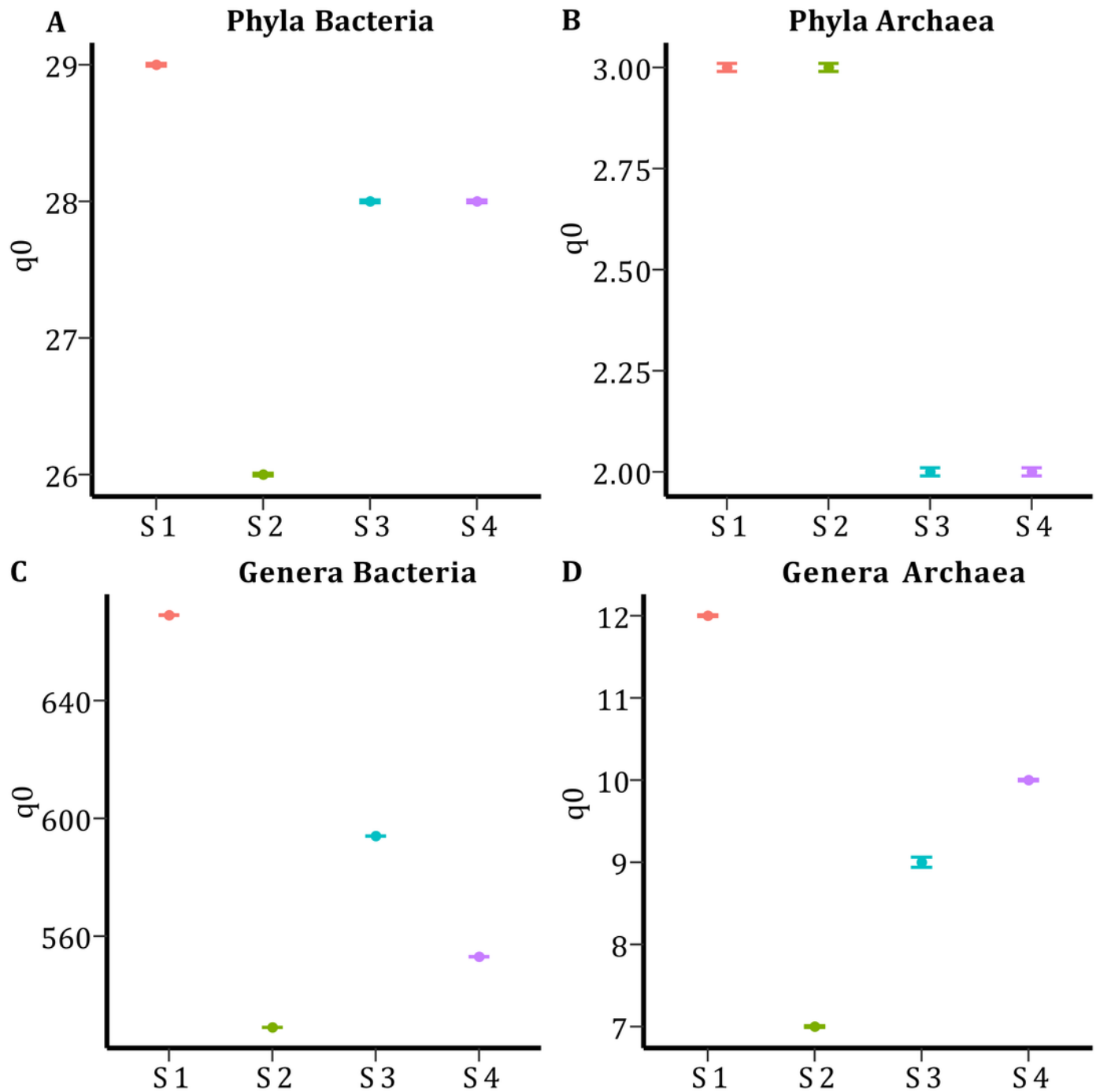
Figure 4

Species Richness (q0) per sample and taxonomic level

A) Species richness for the phyla of the Bacteria domain. B) Species richness for the phyla of Archaea domain. C) Species richness for the genera of the Bacteria domain. D) Species richness for the genera of the Archaea domain

A **Phyla Bacteria**

B **Phyla Archaea**

C **Genera Bacteria**

D **Genera Archaea**
Figure 5

Enzymes annotated in the fecal metagenome of the volcano rabbit

Comparison of enzymes according to their catalytic action: Transferases, Hydrolases, Oxidoreductases, Ligases, Lyases and Isomerases per sample and by number of contigs (COUNTS) using PROKKA

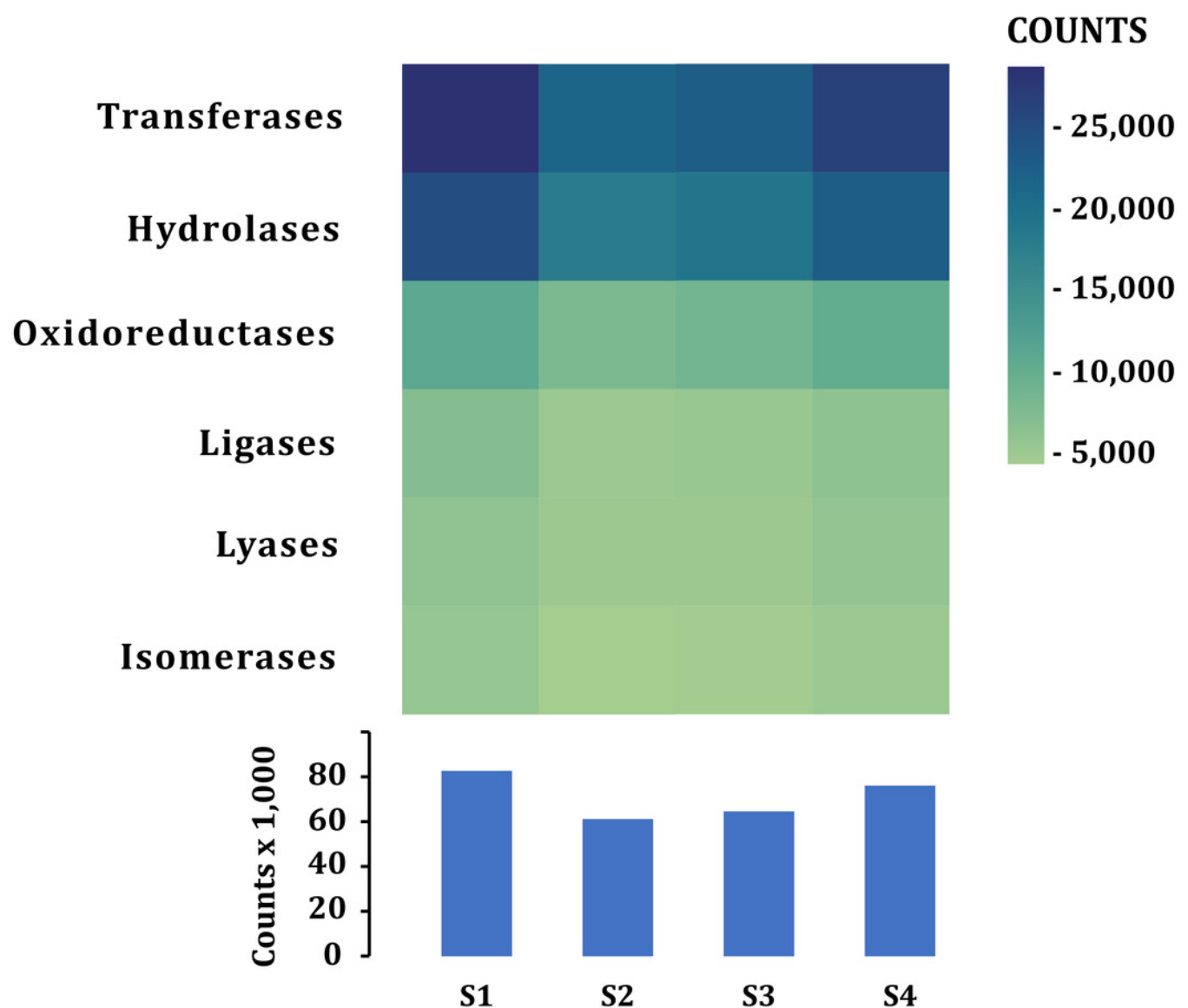



Figure 6

Functional annotation categories in the fecal metagenome of the volcano rabbit

Comparison of categories of functional annotation by sample and by number of contigs (COUNTS) using GhostKOALA

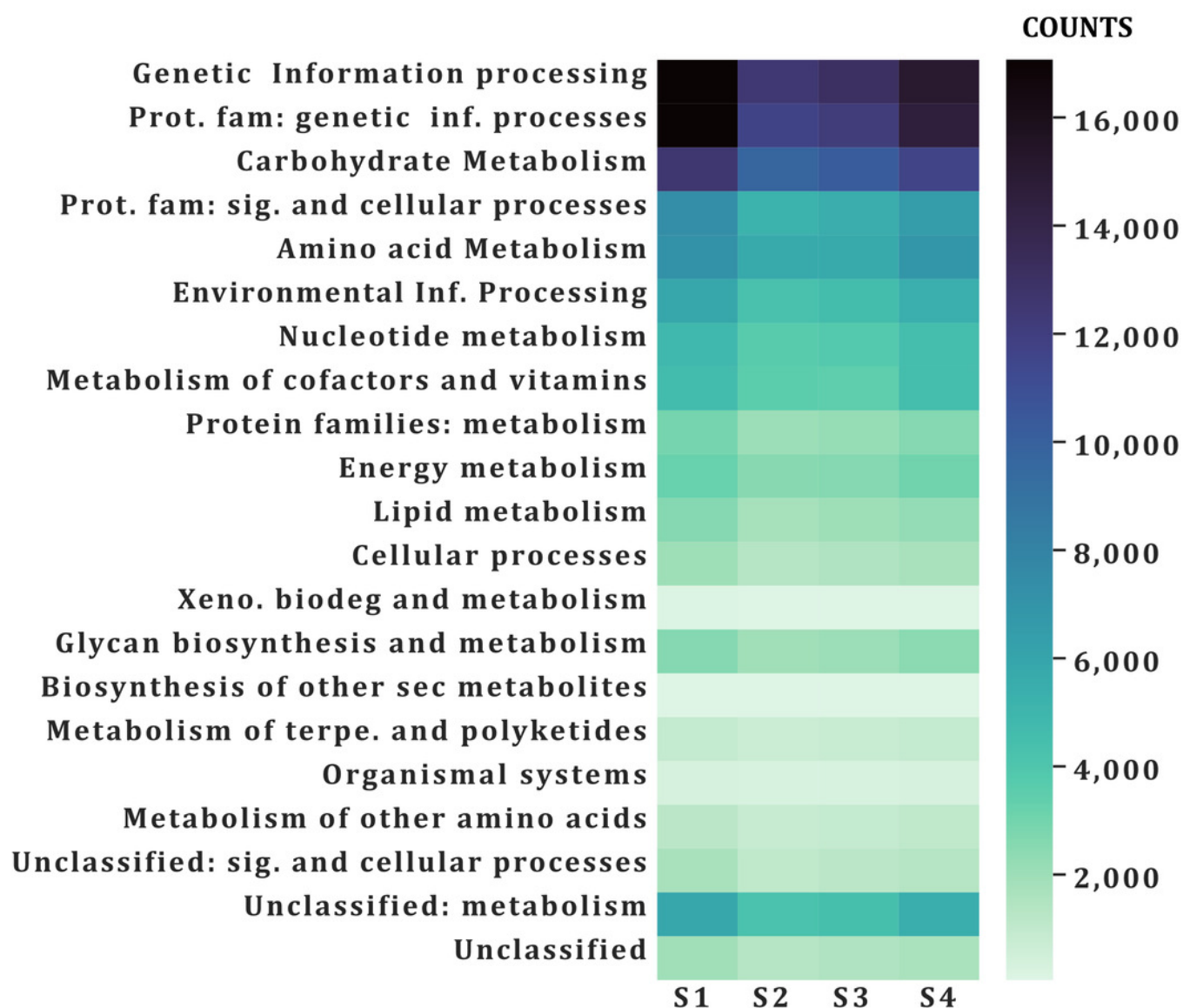
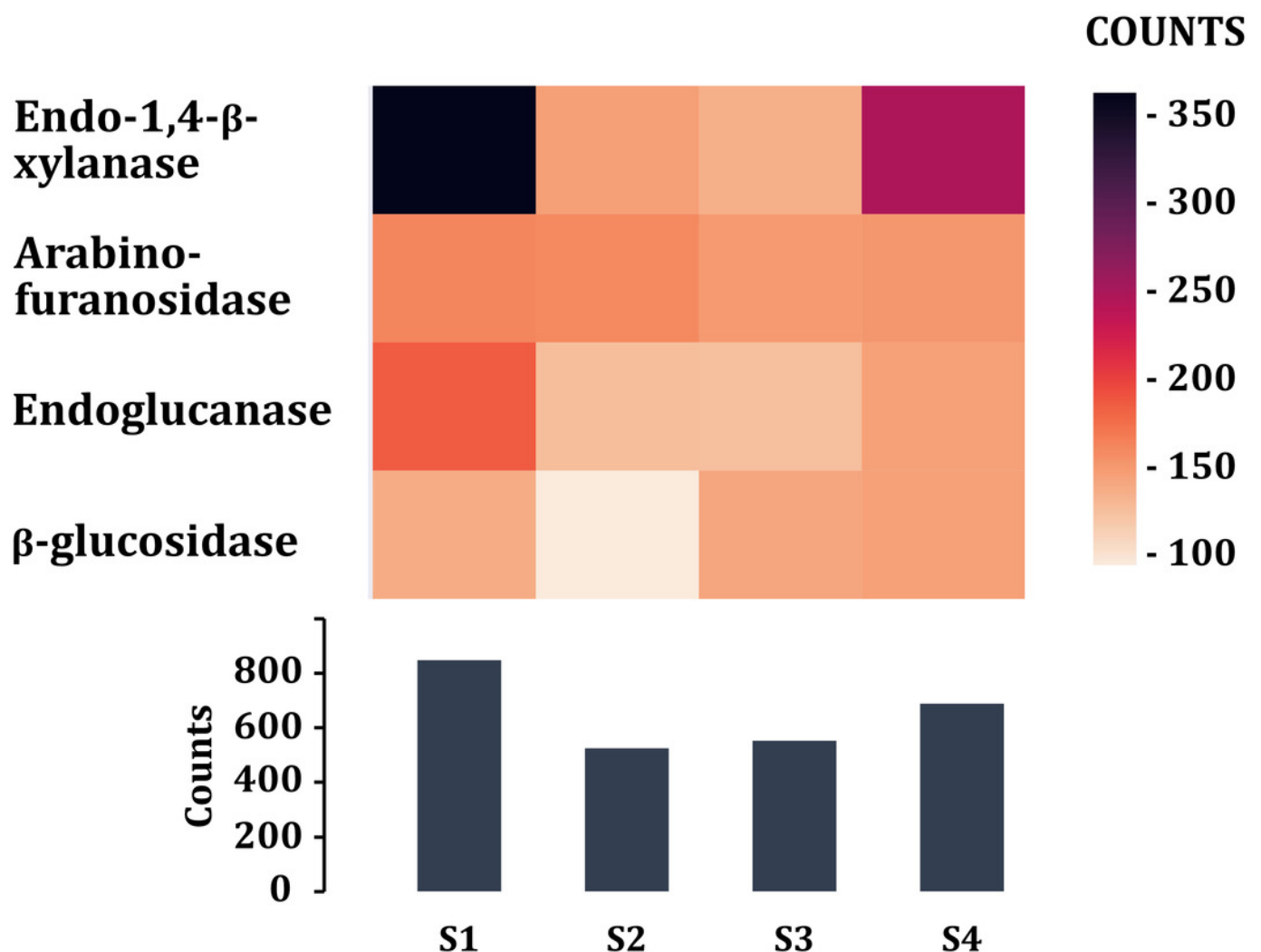



Figure 7

Enzymes (class 3) associated in the degradation of cellulose and hemicellulose of plants

Comparison of the enzymes associated with plant fiber degradation by sample and by number of contigs (COUNTS)

Most abundant phyla, families, and genera in the fecal microbiomes of the volcano rabbit.

Phylum	Family	Genus	Global relative abundance of the genus (%)
Proteobacteria	Moraxellaceae	*Acinetobacter*	2.82
		Moraxella	1.25
		Psychrobacter	1.23
	Pseudomonadaceae	*Azotobacter*	0.87
		Pseudomonas	1.09
	Hafniaceae	*Hafnia*	0.24
		Obesumbacterium	0.20
		Edwardsiella	0.26
	Erwiniaceae	*Erwinia*	0.23
		Pantoea	0.24
	Yersiniaceae	*Serratia*	0.22
	Enterobacteriaceae	*Enterobacter*	0.70
		Citrobacter	0.67
		Klebsiella	0.65
		Escherichia	0.51
		Lelliottia	0.48
		Salmonella	0.50
		Cronobacter	0.46
		Kosakonia	0.38
		Raoultella	0.31
		Pluralibacter	0.35
		Cedecea	0.29
		Leclercia	0.25
		Candidatus Riesia	0.25
Firmicutes	Lachnospiraceae	*Lachnoclostridium*	5.59
		Blautia	3.02
		Roseburia	3.45
		Butyrivibrio	2.61
		Herbinix	1.60
		Cellulosilyticum	1.39
		Anaerostipes	2.04
		Lachnoanaerobaculum	1.83
	Clostridiaceae	*Clostridium*	1.23
		Mordavella	1.16
		Geosporobacter	1.02
		Alkaliphilus	1.12
		C. Arthromitus	1.00
	Oscillospiraceae	*Ruminococcus*	1.00
		Faecalibacterium	1.08
		Oscillibacter	0.74
		Flavonifractor	1.13
		Ethanoligenens	0.96
		Monoglobus	0.79

Functional annotation comparison between all samples

The numbers indicate the total of annotated contigs in each category per sample.

Categories	S1	S2	S3	S4
CDS	131028	96532	101789	119898
tmRNA	90	63	69	87
rRNA	511	387	387	422
tRNA	6389	4850	5098	5430