Diversity structure of the microbial communities in the guts of four neotropical termite species

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The termite gut microbiome is dominated by lignocellulose degrading microorganisms. This study describes the intestinal microbiota of four Argentinian higher termite species with different feeding habits: *Microcerotermes strunckii* (hardwood), *Nasutitermes corniger* (softwood), *Termes riograndensis* (soil organic matter/grass) and *Cornitermes cumulans* (grass) by deep sequencing of amplified 16S rRNA and ITS genes. In addition, we have performed a taxonomic and gut community structure comparison incorporating into the analysis the previously reported microbiomes of additional termite species with varied diets. The bacterial phylum Spirochaetes was dominant in the guts of *M. strunckii*, *N. corniger* and *C. cumulans*, whereas Firmicutes predominated in the *T. riograndensis* gut microbiome. A single bacterial genus, *Treponema* (Spirochaetes), was dominant in all termite species, except for *T. riograndensis*. Both in our own sequenced samples and in the broader comparison, prokaryotic α-diversity was higher in the soil/grass feeders than in the wood feeders. In addition, the β-diversity of prokaryotes and fungi was highly dissimilar among strict wood-feeders, whereas that of soil- and grass- feeders grouped more closely. Ascomycota and Basidiomycota were the only fungal phyla that could be identified in all gut samples, because of the lack of reference sequences in public databases. In summary, higher microbial diversity was evident in termites with more versatile diet preferences, suggesting that diet, along with other factors (*e. g.* host taxonomy) driven microbial community assembly in the termite gut.

1 **Diversity structure of the microbial communities in the guts of four**

2 **Neotropical Termite Species**

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24 **Abstract**

25 The termite gut microbiome is dominated by lignocellulose degrading microorganisms. This

26 study describes the intestinal microbiota of four Argentinian higher termite species with

27 different feeding habits: *Microcerotermes strunckii* (hardwood), *Nasutitermes corniger*

28 (softwood), *Termes riograndensis* (soil organic matter/grass) and *Cornitermes cumulans* (grass)

29 by deep sequencing of amplified 16S rRNA and ITS genes. In addition, we have performed a

30 taxonomic and gut community structure comparison incorporating into the analysis the

31 previously reported microbiomes of additional termite species with varied diets. The bacterial

32 phylum Spirochaetes was dominant in the guts of *M. strunckii*, *N. corniger* and *C. cumulans*,

33 whereas Firmicutes predominated in the *T. riograndensis* gut microbiome. A single bacterial

34 genus, *Treponema* (Spirochaetes), was dominant in all termite species, except for *T.*

35 *riograndensis*. Both in our own sequenced samples and in the broader comparison,

36 prokaryotic α-diversity was higher in the soil/grass feeders than in the wood feeders. In

37 addition, the β-diversity of prokaryotes and fungi was highly dissimilar among strict wood-

38 feeders, whereas that of soil- and grass- feeders grouped more closely. Ascomycota and

39 Basidiomycota were the only fungal phyla that could be identified in all gut samples, because of

40 the lack of reference sequences in public databases. In summary, higher microbial diversity was

41 evident in termites with more versatile diet preferences, suggesting that diet, along with other

42 factors (*e. g.* host taxonomy) driven microbial community assembly in the termite gut.

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53 **Introduction**

54 Termites are extremely efficient in degrading lignocellulose, and may be useful as "bioreactor

55 models" for the conversion of lignocellulosic biomass into biofuels and other biomaterials

56 (*Brune, 2014*).

57   Termites are broadly separated into 'lower' and 'higher' groups. The gut microbiota of

58 lower termites consists of Bacteria, Archaea and Eucarya (such as flagellates and yeasts),

59 whereas higher termites lack flagellated protozoans (*Ni & Tokuda, 2013*). These microbial

60 symbionts have various roles in digestive processes, by participating in multiple functions

61 including carbohydrate and nitrogen metabolism, oxygen and hydrogen consumption, N_2

62 fixation, modifications of aromatic polymers and humification (*Brune, 2014; Santana et al.,*

63 *2015*).

64   All termites feed on lignocellulose, the main component of plant cell walls. Lower

65 termites (families Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae,

66 Rhinotermitidae and Serritermitidae) have specific diets restricted to woody tissue, whereas

67 higher termites (family Termitidae) have diverse feeding habits, which include wood, grass,

68 fungi, lichen, litter, dung, humus and soil. Termitidae is the most diverse family of termites

69 (around 75% of all species). This family comprises eight subfamilies: Apicotermitinae,

70 Cubitermitinae, Foraminitermitinae, Macrotermitinae, Nasutitermitinae, Sphaerotermitinae,

71 Syntermitinae and Termitinae. To date, 80 genera and 458 species, distributed in four

72 subfamilies, Apicotermitinae, Nasutitermitinae, Syntermitinae and Termitinae, have been

73 identified in the Neotropical Region (*Krishna et al., 2013*).

74   The termite gut microbiome was primarily believed to be determined by the host

75 phylogeny, with influence from the diet (*Hongoh, 2010; Rahman et al., 2015; Tai et al., 2015*).

76   More recently, *Mikaelyan et al.* (*2015*) suggested that the diet was the principal determinant of

77   the higher termite gut microbiome composition showing that in all analysis of bacterial

78   community structure, wood-feeding species were clearly separated from humus and soil feeders.

79   Nevertheless, for each feeding source, a grouping of bacterial phylotypes by termite subfamily

80   related to the taxonomy of the host was evidenced (*Mikaelyan et al. 2015*). Furthermore,

81   *Calusinska et al.* (2020) investigated the adaptation of two higher termite colonies

82   (*Cortaritermes* sp.) to *Miscanthus* (a perennial grass) consumption on laboratory conditions and

83   constated the development of a diet-driven, adapted microbial consortium. Most authors agree

84   that insect gut bacterial diversity is determined by environmental behavior, diet, developmental

85   stage and host phylogeny (*Hoback & Stanley, 2001; Yun et al. 2014; Rahman et al. 2015;*

86   *Bourguignon et al., 2018; Dietrich et al., 2014*). However, the relative influence of each of these

87   factors is still not fully elucidated. In line with the above-mentioned studies, *Rahman et al.*

88   (*2015*) concluded that even though the termite gut microbiome is mainly modulated by vertical

89   inheritance, there may be adaptative changes in the microbial populations due to diet. Also,

90   *Dietrich et al.* (2014) showed that phylogeny is not the unique factor influencing the termite

91   microbiota composition, as they observed that changes in the diet or new niches can modify the

92   bacterial community structure. *Bourguignon et al.* (*2018*) stated that termite gut microbiota is a

93   result of a combination of vertical inheritance showing strong host specificity and horizontal

94   transmission, where the latter can occur indirectly through the feeding substrates or via

95   aggressive encounters. In addition, other authors conclude that there is a functional correlation

96   between gut microbiomes from different termite hosts (*Marinowska et al., 2020*). They affirm

97   that each termite species is a unique organism with its own gut microbiome and that there are

98   functional similarities between microbial populations across different termite hosts.

99   In this study, we used prokaryotic 16S rRNA gene and fungal internal transcribed spacer

100   (ITS) sequences to compare the gut microbiota of four higher termite species with different

101   feeding habits and from three different subfamilies *Cornitermes cumulans* (*Syntermitinae*),

102   *Microcerotermes strunckii* (Termitinae, Amitermes group), *Nasutitermes corniger*

103   (*Nasutermitinae*) and *Termes riograndensis* (*Termitinae*, Termes group). *Cornitermes cumulans*

104   builds mounds and feeds mainly on herbaceous material, predominantly grasses, but its diet may

105   include cow dung and degraded wood (*Souza et al., 2017*). The soil-mound building termite *T.*

106   *riograndensis* feeds mainly on soil and plant material. On the other hand, *M. strunckii* and *N.*

107   *corniger* are strict wood -feeders, and therefore consume dry, wet or partially decayed

108   hardwoods and softwoods (*Scheffrahn et al., 2005*). Here, we determined the structure of the

109   bacterial and fungal communities in their guts and we performed a taxonomic and gut

110   community structure comparison among these and other termite species previously reported.

111   This is the first characterization of the gut microbiota of *T. riograndensis* and *M. strunckii*. Thus,

112   this research provides novel information on the gut microbial communities of some unexplored

113   termite species and contributes to shed light on the ecology and evolution of termites and their

114   gut symbionts.

115

116   **Materials & Methods**

117   **Insect collection**

118   The termite species *Cornitermes cumulans*, *Microcerotermes strunckii*, *Nasutitermes corniger*

119   and *Termes riograndensis* are widely distributed in Northeastern Argentina.

120   Specimens of *C. cumulans* (S 28°04′50.2″: W 58°16′12.1″) and *T. riograndensis* (S

121   27°25′29.7″: W 58°38′53.7″) were field-collected in Corrientes province, Argentina, from

122   mounds located in grasslands consisting mainly of *Andropogon lateralis* Nees and *Paspalum*

123   *notatum* Flüggé. *M. strunckii* (S 27°42′43.9″: W 59°13′35.2″) and *N. corniger* (S 27°27′38.3″:

124   W 58°49′19.6″) were sampled in Chaco province from live trees *Myracrodruon balansae* (Engl.)

125   Santin (hardwood) and *Peltophorum dubium* (Spreng.) Taub (softwood), respectively.

126   The termites were collected with the authorization of the Dirección de Recursos Naturales

127   del Ministerio de Turismo de la provincia de Corrientes (permission number 845/13). No

128   endangered or protected species were used in this study. The taxonomic identification of the

129   termite species was inferred by morphology of the digestive tract of the workers caste specimens.

130   Worker specimens were stored at -20°C until further processing.

131

132   **DNA extraction**

133   Worker caste specimens were surface sterilized with 70% ethanol and their whole guts were

134   dissected under a binocular microscope using sterile forceps. Ten dissected whole guts were

135   pooled in a microtube containing RNA-later (Ambion, Grand Island, USA); three independent

136   extractions were performed per termite species. Microbial genomic DNA was extracted from the

137   triplicate gut samples using the DNeasy Blood and Tissue kit (Qiagen, USA) according to the

138   manufacturer's instructions. In order to maximize the disruption of the gut tissues and their

139   content, a thoroughly grinding with plastic pestles was performed prior to the chemical lysis.

140   The V3 and V4 hypervariable regions of the bacterial and archaeal 16S rRNA gene were

141   amplified using the specific barcoded primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′)

142   (*Turner et al., 1999*) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (*Caporaso et al., 2012*).

143   The ITS2 region of the ITS rDNA was amplified using the specific barcoded primers Forward

144   (5′-GCATCGATGAAGAACGCAGC-3′) and Reverse (5′-

145   ATATGTAGGATGAAGAACGYAGYRAA-3′) to assess fungal diversity. The samples were

146   sequenced on an Illumina MiSeq instrument at the Molecular Research DNA (MR DNA)

147   sequencing facility (Shallowater, Texas, USA).

148

149   **Bioinformatic analysis of 16S rRNA and ITS sequences**

150   The paired end short reads were merged into single end reads and demultiplexed using the

151   barcode sequences for each sample. The analyses of the 16S rRNA and ITS sequences were

152   performed in the Qiime2 v2018.6 (https://qiime2.org) (*Bolyen et al., 2019*). Chimera

153   identification and Amplicons Sequence Variants (ASVs) clustering were performed using

154   DADA2 (*Callahan et al., 2016*) plugin in QIIME 2. The sequences were trimmed from the left at

155   35 base pairs (to remove leftover adapter and primer sequences) and truncated at 232 base pairs

156   during the ASV clustering in DADA2. The sequences were assigned to ASVs using SILVA 128

157   16S rRNA (*Quast et al., 2013*) and UNITE (*Abarenkov et al., 2010*) database for

158   Bacteria/Archaea and fungi, respectively.

159   The resulting 16S rRNA representative sequences were aligned using MAFFT aligner

160   (*Katoh et al., 2002*) and an unrooted tree was produced using FastTree 2 (*Price et al., 2010*). The

161   tree was rooted at midpoint for the phylogenetic diversity analysis in QIIME v2. The α- and β-

162   diversity indices for the 16S rRNA were analyzed in the QIIME 2 pipeline. In addition, α-

163   diversity between the termite gut microbiomes was compared using the Kruskal-Wallis test,

164   followed by false discovery rate (FDR) correction in Qiime2 pipeline. Weighted and unweighted

165   UniFrac dissimilarities matrices were also obtained from the QIIME 2 pipeline (*Lozupone et al.,*

166   *2011*).

167   Alpha- and β- diversity indexes for the fungal communities were calculated and applied

168   in the phyloseq (*McMurdie & Holmes, 2013*) and Vegan (*Dixon, 2003*) package in R. The test of

169   significance for the β- diversity of ITS ASVs was performed after Hellinger transformation and

170   Bray-Curtis distance matrix applied for the adonis function in Vegan package. The test of

171   homogeneity of dispersion was performed using the betadisper function and Bray-Curtis

172   dissimilarity in the Vegan package. The analysis of significant differences was performed based

173   on the groups (termite species and feeding source (wood, soil/grass)). A p value < 0.05 was set as

174   the cutoff for significance.

175

176   **Comparison of the results from this study with gut bacterial community taxonomy and**

177   **structures of higher termites previously reported**

178   A broader community structure comparison was performed, including our data and those

179   obtained previously from seven additional termite species (*Bourguignon et al., 2018*; *Mykaelyan*

180   *et al., 2017*) (Table 1).

181   The paired end short reads were merged into single end reads and demultiplexed using

182   the barcode sequences for each sample. The analyses of the 16S rRNA were performed in the

183   Qiime2 v2018.6 (https://qiime2.org) (*Bolyen et al., 2019*). Chimera identification and ASV

184   clustering were performed using DADA2 (*Callahan et al., 2016*) plugin in QIIME 2. The

185   sequences were trimmed 40 bases pairs from left (5') (to remove any leftover adapter/primer

186   sequence) and truncated at 200 base pairs during the DADA2 ASV clustering step. The

187   representative ASV sequences were assigned taxonomy using SILVA 132 16S rRNA (*Quast et*

188   *al., 2013*) database for Bacteria/Archaea. The resulting 16S rRNA representative sequences were

189   aligned using MAFFT aligner (*Katoh et al., 2002*) and an unrooted tree was produced using

190   FastTree 2 (*Price et al., 2010*). The tree was rooted at midpoint for the phylogenetic diversity

191   analysis in QIIME v2. The α- and β- diversity indices for the 16S rRNA were analyzed in the

192 QIIME 2 pipeline. Weighted and unweighted UniFrac dissimilarities matrices were calculated

193 and plotted in phyloseq package (*McMurdie & Holmes, 2013*).

194   The 16S rRNA ASV table was rarefied for 14,042 sequences in 10,736 ASVs (total

195 number of sequences 393,176 for 27 samples). One sample corresponding to *Neocapritermes*

196 *taracua* was removed from the further downstream analyses due low number (8,210 sequences)

197 of ASV sequences. Betadisper test for homogeneity dispersion was performed in the Vegan

198 (*Dixon, 2003*) package in R. The test of significance for the 16S rRNA ASVs was performed by

199 adonis function (strata = location) of Vegan package (*Dixon, 2003*) in R. The analysis of

200 significant differences based on the groups (termite and diet (soil/grass, humus, litter and wood)).

201 A p value < 0.05 was set as the cutoff for significance of the statistical tests.

202

203 **Accession Numbers**

204 The sequences obtained in this study are available in NBBI Sequence Read Archive (Bioproject

205 PRJNA480379). The accession numbers are SRR7503210 (16S rRNA gene) and SRR7503211

206 (ITS).

207

208 **Results**

209 Illumina MiSeq sequencing of 16S rRNA gene and ITS amplicons, derived from gut samples

210 from four termite species generated 1,761,565 high quality sequences. The 16S rRNA ASV table

211 was rarefied to 79,256 sequences resulting in 4,882 ASVs (total number of sequences 951,072

212 for 12 samples). The ITS ASV table was rarefied to 7,418 sequences belonging to 149 ASVs

213 (from a total of 66,762 sequences obtained from nine samples) (Table S1A). In addition, in the

214 comparative analysis of the results from this study with previously works, a total number of

215   sequences 393,176 for 27 samples were included; the 16S rRNA ASV table was rarefied for

216   14,042 sequences in 10,736 ASVs (Table S1B).

217

218   **Bacterial, archaeal and fungal taxonomy**

219   We identified 23 bacterial phyla in the termite guts of the four species tested. The dominant

220   communities in the guts of wood-feeding termites (*M. strunckii* and *N. corniger*) were

221   Spirochaetes (51% to 61%), followed by Fibrobacteres (~13%). Both species also showed

222   similar relative abundances of Bacteroidetes (~8%) and Firmicutes (~8% in the case of *N.*

223   *corniger* and slightly less, ~6%, for *M. strunckii*). Only a few reads (~2%) remained as

224   unclassified bacteria (Fig. 1A). Thus, at the phylum level, gut communities of both wood-feeding

225   species shared highly similar profiles regarding dominant taxa.

226   For the grass-feeding termite *C. cumulans*, Spirochaetes was again the dominant phylum

227   (~44% of the total bacterial community), followed by Firmicutes (~23%) and Bacteroidetes

228   (~13%). By contrast, the dominant phylum in the soil/grass-feeding species *T. riograndensis* was

229   Firmicutes (~32%), followed by Spirochaetes (~20%) and Bacteroidetes (~13%). Only 2% of the

230   sequences remained unclassified (Fig. 1A). Overall, *Treponema* was the most abundant bacterial

231   genus in *M. strunckii* (59.1%), *N. corniger* (58.3%) and *C. cumulans* (42.8%). This genus was

232   also found in *T. riograndensis*, though at a lower relative abundance (17.5%), where other genera

233   such as *Methanimicrococcus* and *Lactococcus* predominated.

234   Of the Archaea, the only two phyla that could be identified, Euryarchaeota and

235   Bathyarchaeota accounted for less than 2% of the reads, except for *T. riograndensis*, in which

236   they represented almost 6% on average.

237   We also compared the gut bacterial and archaeal taxonomy and community structures

238   found in this study with those of seven additional higher termites available in public databases. A

239   total of 35 bacterial/archeal phyla were identified in the termite guts of the 11 species analyzed.

240   Gut communities were dominated by Spirochaetes in the range of 10% to 74%, Firmicutes (4%

241   to 63%), Bacteroidetes (0.2% to 70%) and Fibrobacteres (0.9% to 32%). Less abundant, though

242   still well represented were Proteobacteria (<21%) and Actinobacteria (<19%), among others

243   (Fig. S1). A little fraction of the reads could not be assigned to any phylum. As for the other

244   phyla, the relative abundance of the Spirochaetes was variable within and between the diet

245   groups. However, the highest abundance was observed in the wood-feeding termite group (up to

246   74%) followed by the soil- and/or grass-feeding group (up to 49%) and the litter-feeder termites

247   *Cornitermes* sp. (consistently ~45%). Termites that feed on soil and/or grass, and humus showed

248   high relative abundance of the phylum Firmicutes (up to ~55% for both groups). One of the

249   triplicates of the wood-feeder *M. parvus* also exhibited an unexpectedly high proportion of

250   Firmicutes, but this was not corroborated in any of the other two replicates. At the genus level

251   most dominant taxa were Termite *Treponema* cluster followed by *Treponema* sp. regardless of

252   the diet group (Fig. S2). For the ITS analysis, *C. cumulans* was excluded because of the low

253   number of reads obtained. Regarding the other three termite species, the absence of matches with

254   the available sequence data in UNITE (~81%, ~93% and ~99% of unclassified reads for *N.*

255   *corniger*, *T. riograndensis*, and *M. strunckii*, respectively) did not allow the taxonomic

256   placement of most of the fungal ASVs. The few taxa identified were assigned to the phyla

257   Ascomycota and Basidiomycota, and within the former, the class Eurotiomycetes was the most

258   abundant (Fig. 1B).

259   A rarefaction analysis performed for each gut sequence dataset retrieved rarefaction

260   curves that reached a plateau for all samples, except for *C. cumulans* ITS sequences. This result

261   suggests that the sample size was large enough to represent the bacterial and fungal diversity

262   present in the communities (Fig. 2).

263

264   **Diversity of prokaryotic and fungal taxa**

265   The prokaryotic diversity of termite guts was analyzed using α- and β-diversity indices. The

266   indices, Shannon, Pielou's evenness and number of observed ASVs, showed no significant

267   differences in α-diversity between the four newly reported termite microbiomes (Fig. 3). In

268   addition, we evaluated the α-diversity between diet groups (soil/grass or strictly grass-feeders,

269   humus -feeders, litter -feeders, and wood -feeders) incorporating sequence data from public

270   databases (Fig. 4). Soil/Grass or strictly grass diet group showed highest number of observed

271   ASVs, however, the comparison was only significantly different for the diet groups soil/grass-

272   humus (Krukal-Wallis; H=6.3, *p*=0.011, *q*=0.035) and soil/grass-wood (Kruskal-Wallis; H=7.7,

273   *p*=0.005, *q*=0.031) (Fig. 4C). The Pielou's-evenness indices showed significant differences

274   between the soil/grass-humus (H=8.0, *p*=0.004, *q*=0.008) and soil/grass-wood (H=9.2, *p*=0.002,

275   *q*=0.007) groups. Also, a significant difference was observed between the humus-litter (H=5.0,

276   *p*=0.025, *q*= 0.038) and humus-wood (H=10.0, *p*=0.001, *q*=0.007) groups (Fig. 4B).

277   The prokaryotic β-diversity of the termite gut microbiome was compared using the

278   unweight UniFrac distances. The gut microbiome composition of the polyphagous soil/grass

279   feeders was found to be similar and grouped distantly from that of the wood feeders, which were,

280   in turn, separated from each other (Fig. 5A). The PERMANOVA test of Unifrac distances

281   revealed that the β-diversity was significantly different for the four termite species, with a

282   marked variation according to the feeding habit (diet group of termites) (strict wood-feeders and

283   soil/grass -feeders) ($p <0.05$) (Table 2). Furthermore, the prokaryotic β-diversity of the new and

284   database-retrieved termite gut microbiomes were compared using the weighted and unweighted

285   UniFrac distances (Table 2). The NMDS of Unifrac distances did not show grouping of the

286   samples in the ordination plot based on the diet groups (Fig 5B-C). The dissimilarity between the

287   termite gut prokaryotic compositions for the diet group was compared using the betadisper (to

288   test the homogeneity dispersion) and adonis (to test the similarity between the prokaryotic

289   communities). The distance to group centroids based on Bray-Curtis distance were significantly

290   different for diet groups (Betadisper, Permutest F=19.607, $p= 0.001$). Adonis also showed

291   significant differences ($F_{(2.646)}= 0.25$, $p= 0.001$) in the community composition between the diet

292   group of termites.

293   The analysis of the fungal community structure was restricted to our novel sequence data,

294   since such information is still lacking in public databases. The Shannon and Pielou's evenness α-

295   diversity indices of fungal communities from *M. strunckii*, *N. corniger* and *T. riograndensis* gut

296   samples differed significantly (Fig. S3). However, the number of observed ASVs showed no

297   significant differences in α-diversity. Fungal α-diversity according to the diet group was also

298   significantly different for the Shannon and Pielou's evenness indices but not for the number of

299   observed ASVs (Fig. S4).

300   To visualize overall similarities and differences in fungal community structure, we

301   calculated Bray-Curtis distances between *M. strunckii*, *N. corniger*, and *T. riograndensis*, and

302   displayed these analyses in the form of two-dimensional NMDS plots (Fig. S5). These analyses

303   revealed that the fungal community composition in the gut samples of the three termite species

304   was significantly different, whereas replicates of the same species were almost identical (Fig.

305   S5). PERMANOVA analysis confirmed significant differences in the fungal communities of the

306   different termite species and diet groups (Table 2).

307

308   **Core microbiome**

309   In total, 38 bacterial ASVs were shared across the four higher termite species reported here;

310   which represent 28.5% of all the obtained sequences (Fig. 6A). Furthermore, the termite gut

311   samples grouped according to their feeding habits, shared 23 (wood feeders) and 38 (soil/grass

312   feeders) additional ASVs (Fig. 6A). However, it has to be noted that *N. corniger* and *T.*

313   *riograndensis*, which differ strongly in their feeding source and belong to distinct subfamilies,

314   shared 31 additional ASVs

315   Of the 38 ASVs, 18 were assigned to *Treponema* sp., which represented 19% of the core

316   microbiome sequences obtained (Fig. S6). These core ASVs were present in higher relative

317   abundances in the guts of *C. cumulans*, *M. strunckii* and *M. corniger* than in the guts of *T.*

318   *riograndensis* (Table S2; Fig S7).

319   The analysis of core prokaryotic communities incorporating a larger number of host

320   species (*i. e. A. meridionalis*, *T. hospes*, *Cornitermes* sp., *Microcerotermes* sp., *M. parvus* and

321   *Nasutitermes* sp.) revealed no shared ASVs between the four diet groups of termites (Fig 6B).

322   However the soil/grass-wood groups shared 188 ASVs. A high number of ASVs were unique to

323   the termite species included in each diet group (soil/grass or grass: 3,729; humus: 2,046; liter:

324   1,285; wood: 3,379) (Fig 6B).

325   We identified 11 fungal core ASVs, which represented an average of 71.3% of all the

326   identified fungal sequences, shared in the gut samples of *M. strunckii*, *N. coringer* and *T.*

327 *riograndensis* (Fig. 6C). However, the most abundant shared ASVs could not be assigned to any

328 taxonomic level (Table S2).

329

330 **Discussion**

331 Numerous studies on the gut microbiota of higher termites have been published in the last two

332 decades (*Warnecke et al., 2007; Otani et al., 2014; Mikaelyan et al., 2015; Rahman et al., 2015;*

333 *Santana et al., 2015; Su et al., 2016, Bourguignon et al*., 2018). However, in a few proportion

334 such studies have dealt with the gut community composition in relation to the feeding habits of

335 the host termite species.

336   This study provides a description of the intestinal microbiota associated with four

337 Argentinian higher termite species (*C. cumulans*, *M. strunckii*, *N. corniger* and *T. riograndensis*),

338 performed by high-throughput 16S rRNA gene and ITS amplicon sequencing analyses. Although

339 data exist on the bacterial microbiota of *C. cumulans* and *N. corniger* (*Dietrich et al., 2014*;

340 *Köhler et al., 2012*; *Warnecke et al., 2007*; *Burnum et al., 2011*; *Mikaelyan et al., 2014*; *Costa et*

341 *al., 2013*, *Grieco et al., 2013*; *2019*); this is the first characterization of the gut microbiota of *T.*

342 *riograndensis* and *M. strunckii*. Furthermore, very little is known about the fungal community in

343 the microbiome of these and other termite species. These termites have different diet preferences

344 ranging from hard- and softwood, to herbaceous materials and, soil/grass. Compared to

345 softwood, hardwood harbors higher amounts of carbon content consisting on cellulose,

346 hemicellulose and low proportion of lignin (*Demirbas, 2005*). Herbaceous plant materials have

347 higher nutritious contents and lower lignin than wood, whereas soil contains diverse organic

348 matter that is selectively utilized by the termite species. We explored the gut microbiota

349 composition of the above-mentioned Argentinian higher termite species taking into consideration

350 their different diet preferences.

351   Overall, Spirochaetes, followed by Fibrobacteres, Bacteroidetes and Firmicutes were the

352 dominant gut phyla in termites feeding on wood or grass (*M. strunckii*, *N. corniger* and *C.*

353 *cumulans*). By contrast, the dominant phylum in the soil/grass feeder *T. riograndensis* was

354 Firmicutes, followed by Spirochaetes and Bacteroidetes. In accordance with our study, other

355 researchers have reported Spirochaetes as one of the most abundant phyla in wood- and grass-

356 feeding higher termite guts (*Hongoh et al., 2005; Warnecke et al., 2007; Köhler et al., 2012;*

357 *Brune, 2014; Dietrich et al., 2014; Mikaelyan et al., 2015; Rahman et al., 2015*). In the last years

358 the majority of glycosyl hydrolase genes encoding putative cellulases and hemicellulases,

359 identified by metagenomic and metatranscriptomic studies, have been associated with

360 Spirochaetes, Fibrobacteres, Bacteroidetes and Firmicutes (*Romero Victorica et al., 2020*; *Ben*

361 *Guerrero et al., 2015*; *Warnecke et al., 2007*; *He et al., 2013*; *Grieco et al., 2019*; *Marynowska*

362 *et al., 2020*; *Calusinska et al., 2020*).

363   The high abundance of Spirochaetes (including the genus *Treponema* sp.), Fibrobacteres

364 and Bacteroidetes in wood-feeding termites may be related to the nitrogen fixation and

365 lignocellulosic processes (*Lilburn et al., 2001; Breznak, 2002; Warnecke et al., 2007; Yamada et*

366 *al., 2007; Su et al., 2016*). In addition, high abundance of Firmicutes (mostly Ruminococcacae)

367 in *T. riograndensis* was in accordance with previous reports of soil- and humus- feeding termites

368 (*He et al., 2013; Dietrich et al., 2014; Mikaelyan et al., 2015; Santana et al., 2015*).

369   In particular, several studies have reported a similar proportion of Spirochaetes

370 (approximately 50 to 60% of total prokaryotic population) in the gut microbiome of *N. corniger*

371 (*Warnecke et al., 2007*; He *et al., 2013*; Dietrich *et al., 2014*; Santana *et al., 2015*; Su *et al.,*

372   *2016*). However, *Köhler et al.* (2012) observed a lower proportion of Spirochaetes in *N. corniger*

373   and *N. takasagoensis*. This discrepancy could be due to variations in DNA extraction methods

374   (*Morgan et al., 2010*) and/or the use of different PCR oligonucleotides (*Engelbrektson et al.,*

375   *2010*).

376   A low proportion of Archaea was present in the profile of higher termite gut

377   communities. The detected archaeal phyla were Euryarchaeota and Bathyarchaeota.

378   Euryarchaeota includes closely related genera already known for their methanogenic activity

379   (*Rahman et al., 2015*). The methanogenic archaeon *Methanimicrococcus* sp. was present in the

380   core microbiome albeit in different proportions. This genus has been detected in the gut

381   microbiomes of other higher termites and cockroaches (*Paul et al., 2012*).

382   The extremely high relative abundance of *Treponema* spp. (phylum Spirochaetes) in the

383   core of *C. cumulans*, *M. strunckii* and *N. corniger*, and at a lower extent in that of *T.*

384   *riograndensis*, suggests that *Treponema* genus has an important role on the overall physiology

385   and digestive processes of wood- and grass- feeding higher termites. The predominance of

386   *Treponema* in the termite gut microbiota has been described in several studies (*Warnecke et al.,*

387   *2007; Köhler et al., 2012; Shi et al., 2013; Benjamino & Graf, 2016*). Thus, the differences in

388   their core microbiomes might be related to the phylogeny of the termite and also, to their diet

389   habits. In order to infer possible relationships between gut microbial community, phylogeny, and

390   diet; the gut communities of additional termite species reported elsewhere have been included for

391   a broader comparison. Again, Spirochaetes, Firmicutes, Bacteroidetes and Fibrobacteres were the

392   dominant phyla. Also the most dominant taxa were Termite *Treponema* cluster followed by

393   *Treponema* sp.

394   In the four species evaluated in this study, the diversity of the fungal community was

395   markedly lower than that of prokaryotes. The high proportion of taxonomically unclassified

396   fungal ASVs may result from the lack of representative sequences in the UNITE database

397   (*Hongoh, 2010; Santana et al., 2015*). Although fungi are not as prevalent as bacteria in higher

398   termite guts, an important unresolved issue is to determine the role of fungi in cellulolytic

399   processes development and fitness. Some of these functions could be to provide a nitrogen

400   source, degrade high molecular weight molecules and produce pheromones for mating and

401   communication (*de León et al., 2016; Zhang et al., 2018*). However, the role of fungal

402   microbiota in these processes is not clear yet (*Brune, 2014*).

403   The fungi classes that could be identified in the core were Eurotiomycetes and

404   Malasseziomycetes. Eurotiomycetidae are producers of secondary metabolites, fermentation

405   agents and xerophile and psychrophile enzymes. They had been previously reported in the gut of

406   the litter-feeding termite *Synthermes wheeleri* (*Santana et al., 2015*). Malasseziomycetes are

407   ecologically diverse and wide spread yeasts. The genus Malassezia includes lipophilic yeasts and

408   has been known as a common inhabitant of human skin (*Paulino et al., 2008*). A report by *Zhang*

409   *et al.* (*2003*) also identified this yeast in the guts of beetles.

410   The presence of a common core microbiota suggests that these taxa are retained despite

411   differences in habitat, geography and food source, and regardless of host phylogeny. This core

412   composition may be important for the maintenance key functions and may serve as the basis for

413   microbial community resistance and/or resilience (*Huse et al., 2012; Shade & Handelsman,*

414   *2012; Benjamino & Graf, 2016*). However, when sequences obtained from more termite species

415   were added to the analysis, no shared ASVs between all four diet groups of termites was evident,

416   suggesting that both host termite phylogeny and diet (and eventually other additional factors) can

417   influence the community structures of gut microbiota.

418   The α-diversity of gut bacterial communities in the soil/grass feeder group was

419   significantly higher than that in wood feeders. Among the former group, whereas the gut

420   microbiota in *T. riograndensis* (a soil- and grass- feeding termites) was higher than in species

421   that fed on grass only (*C. cummulans*). The lower microbial diversity found in the wood- feeding

422   termites may be related to the maintenance of a more specialized microbiota that is necessary for

423   performing an efficient lignocellulose metabolism, and therefore for the host survival (*Breznak*

424   *& Brune,* 1994*; Colman et al., 2012*). The studied species of wood- feeding termites have a very

425   limited diet, which includes complex carbohydrates (cellulose, hemicellulose and lignin), and

426   this characteristic may explain the lower α-diversity. Feeding on live trees may expose the host

427   and potentially its microbiota to tree physiological responses (*Morewood et al., 2004*), which

428   may further shape gut community dynamics. The high α-diversity in the soil/grass feeding

429   termites could be related to the diverse range of carbon and nitrogen sources available in their

430   diets; as more complex substrates require more complex degradative capacity and therefore more

431   complex communities. On the other hand, the host habitat also may influence the relative

432   bacterial abundances of the termite gut microbiota (*Yun et al., 2014*). When including in the

433   analysis previously reported sequences obtained from additional termite species, the bacterial α-

434   diversity indices (Shannon and Pielou's-evenness) and number of observed ASVs found in the

435   soil/grass or strictly grass diet group were, again, significantly higher than in wood-feeders.

436   Significant differences were also evidenced for some indices between the other feeding groups

437   suggesting that a number of factors, which may include diet and host taxonomy, contribute to

438   shape the gut microbiota.

439   The intercommunity analysis restricted to our four Argentinian termite species showed

440   that the gut microbiomes of soil/grass feeders were clearly separated from those of wood feeders.

441   Soil/grass-feeding termite species grouped closely, whereas the wood -feeders were spatially

442   separated from each other. The replicates of *C. cumulans*, *T. riograndensis* and *M. strunckii*

443   showed little variation, whereas those of the wood feeder *N. corniger* were more disperse. The

444   termites *M. strunckii* and *N. corniger* were sampled from alive trees of *Myracrodruon balansae*

445   (hardwood) and *Peltophorum dubium* (softwood). Even though the relative abundance at the

446   phylum level was similar, microbial species composition was different between both termite

447   species.

448   The β-diversity analysis including gut microbiome previously reported showed non-

449   significant clustering of the termites for the diet groups. Test of homogeneity dispersion using

450   betadisper suggested that the sample groups are having significantly low variance in the

451   community dispersal ($p < 0.001$). PERMANOVA test using the adonis function also suggested

452   the significant differences in the termite gut community using the diet as a grouping factor.

453   The understanding of the termite-microbiome interaction requires the exploration of the

454   composition and structure of the microbiota, as well as the characterization of its main metabolic

455   activities in different taxonomic termite groups with different types of diets. Altogether, and

456   concerning the four Argentinian termite species, no obvious pattern was observed in the

457   microbial community structures, except for the similar relative abundance of bacterial phyla in

458   the case of strict wood- feeders. This provides further evidence that the gut microbiota

459   composition is the result of multiple factors, which may include (but not be limited to) diet and

460   host taxonomy.

461

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471

472 **Author Contributions**

473 Surendra Vikram performed the experiments, analyzed the data, prepared figures and tables,

474 authored or reviewed drafts of the paper, and approved the final draft.

475 Joel D. Arneodo conceived and designed the experiments, performed the experiments, analyzed

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481 the final draft.

482 Clara Etcheverry performed the experiments, analyzed the data, reviewed drafts of the paper, and

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484   Don A Cowan analyzed the data, authored and reviewed drafts of the paper, and approved the

485   final draft.

486   Paola Talia conceived and designed the experiments, performed the experiments, analyzed the

487   data, prepared figures and/or tables, authored and reviewed drafts of the paper, and approved the

488   final draft.

489

490   **Tables and Figures titles**

491   **Table 1**: Summary of the higher termites used in the current study, their taxonomy classification,

492   feeding groups and accession numbers of bacterial 16S rRNA amplicon libraries.

493   **Table 2**: PERMANOVA analysis of bacteria/archaea and fungi.

494   **Fig 1**. Relative abundance of bacteria, archaea (phyla level) and fungi (class level) in the gut of

495   Neotropical termites.

496   **Fig 2**. Rarefaction curves for the prokaryotic and fungi observed ASVs at 97% sequence identity.

497   **Fig 3**. Prokaryotic α-diversity measures (A) Shannon, (B) Pielou's evenness and (C) number of

498   observed ASVs of the four species of termites. Comparisons were performed using the Kruskal-

499   Wallis followed by *Benjamini & Hochberg* FDR correction.

500   **Fig 4.** Prokaryotic α-diversity measures.

501   **Fig. 5**. Beta-diversity NMDS plot of Unifrac distances.

502   **Fig 6**. Venn diagram showing the distribution of shared ASVs across the termite gut.

503

504   **Tables and Figures legends**

505   **Table 1**:

506   *Termites collected in this study.

507   ªBased on the food types given for termite genera (*Jones & Eggleton, 2011*).

508   [b]Based on the dietary information in *Souza et al.*, (2017).

509   [c]Based on dietary information in *French & Ahmed* (2011).

510   [d]Based on observations of *Gontijo & Domingos* (1991).

511   **Table 2**: The statistical analysis of ITS sequence data were performed based on the Hellinger

512   transformation and Bray-Curtis distance-based dissimilarity matrix.

513   **Fig 1**. (A) 16S rRNA gene (B) ITS sequence based taxonomic distribution in triplicate gut

514   samples.

515   **Fig 2**. (A) 16S rRNA gene sequences of *C. cumulans*, *M. strunckii*, *N. corniger* and *T.*

516   *riograndensis*. (B) ITS sequences. (C) 16S rRNA gene of the total ASVs and sequence counts

517   showing the sufficient coverage at the 14,042 sequences per sample.

518   **Fig. 4**. (A) Shannon, (B) Pielou's evenness and (C) number of observed ASVs of the total

519   species of higher termites used to compare between diet groups. Comparisons were performed

520   using the Kruskal-Wallis followed by *Benjamini & Hochberg* FDR correction. The H stats, *p*

521   value and corrected *p* value (*q*) is written on the top of the paired comparison between the diet

522   groups.

523   **Fig 5**. (A) Unweighted Unifrac distance plot. The red, blue, green and orange dots represent the

524   gut microbiome of *C. cumulans*, *M. strunckii*, *N. corniger* and *T. riograndensis*, respectively.

525   The circle and triangle denote the soil/grass and wood feeders, respectively. The first and second

526   principal components explain 34% (PC1) and 24% (PC2) of variations, respectively. (B)

527   Unweighted Unifrac distance of a larger number of host species (C) Weighted Unifrac distance

528   of a larger number of host species.

529   **Fig 6**. (A) Shared prokaryotic ASVs between four gut microbiomes (B) Shared prokaryotic

530   ASVs between four gut microbiomes (B) Shared prokaryotic ASVs between diet groups of

531   termites and (C) Shared fungal ASVs between the three termites.

532

533   **Supporting information**

534   **Fig S1.** Taxonomy plot for the termite samples grouped into diet groups. The plot is showing the

535   relative abundance of the prokaryotic phyla in the gut of termites.

536   **Fig S2.** Taxonomy stack bar-plot of the prokaryotic communities in the diet group. (A) Phylum

537   level taxonomic classification (B) Genus level taxonomic classification. Taxa less than 2% were

538   combined and named as a "<2% abund.".

539   **Fig S3.** Prokaryotic α-diversity measures (Shannon, Pieolou's evenness and number of observed

540   ASVs) according to diet groups (strict wood-feeders versus soil/grass- feeders). Comparisons

541   were performed using the Kruskal-Wallis followed by FDR method in Qiime2.

542   **Fig S4**. Fungal α-diversity measures (Shannon, number of observed ASVs and Pieolou's

543   evenness) in three species of termites. Comparisons were performed using Kruskal-Wallis

544   followed by FDR method in Qiime2.

545   **Fig S5**. Fungal α-diversity measures (Shannon, number of observed ASVs and Pieolou's

546   evenness) according to diet groups (strict wood-feeders versus soil/grass- feeders). Comparisons

547   were performed using Kruskal-Wallis followed by FDR method in Qiime2.

548   **Fig S6**. NMDS plot for the three termite gut samples using Bray Curtis analysis for ITS

549   sequences.

550   **Fig S7**. Relative abundance of 38 core ASVs at classified taxa level in the gut microbiome of *C.*

551   *cumulans*, *M. strunckii*, *N. corniger* and *T. riograndensis*. Redder and lighter red indicate greater

552   and less abundance, respectively.

553 **Table S1**. Number of input and final number of reads for the 16S rRNA and ITS sequence data

554 (S1A). Number of input and output 16S rRNA sequences obtained in the comparative analysis

555 (S1B).

556 **Table S2**: Relative abundance (%) of *C. cumulans*, *M. strunckii*, *N corniger* and *T. riograndensis*

557 core microbiota.

558

559 **References**

560 Abarenkov K, Henrik Nilsson R, Larsson KH, Alexander IJ, Eberhardt U, Erland S, Høiland K,

561 Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Ursing BM, Vrålstad T,

562 Liimatainen K, Peintner U, Kõljalg U. 2010. The UNITE database for molecular identification of

563 fungi - recent updates and future perspectives. *New Phytologist* 186: 281–285. DOI:

564 10.1111/j.1469-8137.2009.03160.x.

565 Ben Guerrero E, Arneodo J, Bombarda Campanha R, Abrão de Oliveira P, Veneziano

566 LabateMT, Regiani Cataldi T, Cataldi A, Labate CA, Martins Rodrigues C, Talia P. 2015.

567 Prospection and evaluation of cellulolytic and hemicellulolytic enzymes using untreated and

568 pretreated biomass in two argentinean native termites. *PLoS One* 10:e0136573. DOI:

569 10.1371/journal.pone.0136573.

570 Benjamino J, Graf J. 2016. Characterization of the Core and Caste-Specific Microbiota in the

571 Termite, *Reticulitermes flavipes*. *Frontiers in Microbiology* 7: 171. DOI:

572 10.3389/fmicb.2016.00171.

573 Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. 2019.

574 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.

575 *Nature Biotechnology*. 37(8): 852-7. DOI: 10.1038/s41587-019-0209-9.

576 Bourguignon T, Lo N, Dietrich C, Šobotník J, Sidek S, Roisin Y, Brune A, Evans TA. 2018.

577 Rampant Host Switching Shaped the Termite Gut Microbiome. *Current Biology* 28(4): 649–

578 654.e2. DOI: 10.1016/j.cub.2018.01.035.

579 Breznak JA, Brune A. 1994. Role of microorganisms in the digestion of lignocellulose by

580 termites. *Annual Review of Entomology* 39: 453–487. DOI:

581 10.1146/annurev.en.39.010194.002321.

582 Breznak NA. 2002. Phylogenetic Diversity and Physiology of Termite Gut Spirochetes.

583 *Integrative and Comparative Biology* 42: 313–318. DOI: 10.1093/icb/42.2.313.

584 Brune A. 2014. Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews*

585 *Microbiology* 12: 168–180. DOI: 10.1038/nrmicro3182.

586 Callahan BJ, McMurdie PJ, Rosen M, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-

587 resolution sample inference from Illumina amplicon data. *Nature Methods* 13: 581–583. DOI:

588 10.1038/nmeth.3869.

589 Calusinska M, Marynowska M, Bertucci M, Untereiner B, Klimek D, Goux X, Sillam-Dussès D,

590 Gawron P, Halder R, Wilmes P, Ferrer P, Gerin P, Roisin Y, Delfosse P. 2020. Integrative omics

591 analysis of the termite gut system adaptation to Miscanthus diet identifies lignocellulose

592 degradation enzymes. *Communications Biology* 3(1): 275. DOI: 10.1038/s42003-020-1004-3.

593 Caporaso JG, Lauber CL,Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J,

594 Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012.Ultra-high-throughput

595 microbial community analysis on the illumina HiSeq and MiSeq platforms. *ISME Journal* 6,

596 1621−1624. DOI: 10.1038/ismej. 2012.8

597   Colman DR, Toolson EC, Takacs-Vesbach CD. 2012. Do diet and taxonomy influence insect gut

598   bacterial communities?. *Molecular Ecology* 20: 5124–5137. DOI: 10.1111/j.1365-

599   294X.2012.05752.x.

600   Costa PS, Oliveira PL, Chartone-Souza E. Nascimento AMA. Phylogenetic diversity of

601   prokaryotes associated with the mandibulate nasute termite *Cornitermes cumulans* and its

602   mound. 2013. *Biology and Fertility of Soils* 49, 567–574 (2013). DOI: 10.1007/s00374-012-

603   0742-x.

604   de León AV-P, Sanchez-Flores A, Rosenblueth M, Martínez-Romero E. 2016. Fungal

605   community associated with *Dactylopius* (Hemiptera: Coccoidea: Dactylopiidae) and its role in

606   uric acid metabolism. *Frontiers in Microbiology* 7: 954. DOI: 10.3389/fmicb.2016.00954.

607   Demirbas A. 2005. Estimating of structural composition of wood and non-wood biomass

608   samples. *Energy Sources* 27: 761–767. DOI: 10.1080/00908310490450971.

609   Dietrich C, Köhler T, Brune A, Kohler T, Brune A. 2014. The cockroach origin of the termite

610   gut microbiota: patterns in bacterial community structure reflect major evolutionary events.

611   *Applied and Environmental Microbiology* 80(7): 2261–2269. DOI: 10.1128/AEM.04206-13.

612   Dixon P. 2003. VEGAN, a package of R functions for community ecology. *Journal of*

613   *Vegetarian Science* 14: 927–930. DOI: 10.1111/j.1654-1103.2003.tb02228.x.

614   Engelbrektson A, Kunin V, Wrighton KC, Zvenigorodsky N, Chen F, Ochman H, Hugenholtz P.

615   2010. Experimental factors affecting PCR-based estimates of microbial species richness and

616   evenness. *The ISME Journal* 4: 642–647. DOI: 10.1038/ismej.2009.153.

617   French JRJ, Ahmed BM. 2011. Biomimicry of Termite Social Cohesion and Design to Inspire

618   and Create Sustainable System. www.Intechopen.com. DOI:10.5772/19350.

619 Gontijo TA, Domingos DJ. 1991. Guild distribution of some termites from cerrado vegetation in

620 south-east Brazil. *J Trop Ecol* 7:523–9.

621 Grieco MB; Lopes FAC, Oliveira LS, Tschoeke DA, Popov, CC, Thompson CC, Gonçalves LC,

622 Constantino R, Martins OB, Kruger RH, de Souza W,Thompson FL. 2019. Metagenomic

623 Analysis of the Whole Gut Microbiota in Brazilian Termitidae Termites *Cornitermes cumulans,*

624 *Cyrilliotermes strictinasus*, *Syntermes dirus*, *Nasutitermes jaraguae*, *Nasutitermes aquilinus*,

625 *Grigiotermes bequaerti*, and *Orthognathotermes mirim*. *Current Microbiology* 76(6):687-697.

626 DOI: 10.1007/s00284-019-01662-3.

627 Grieco MA, Cavalcante JJ, Cardoso AM, Vieira RP, Machado EA, Clementino MM, Medeiros

628 MN, Albano RM, Garcia ES, de Souza W, Constantino R, Martins OB. 2013. Microbial

629 community diversity in the gut of the South American termite *Cornitermes cumulans* (Isoptera:

630 Termitidae). *Microbial Ecololgy*. 65(1):197-204. DOI: 10.1007/s00248-012-0119-6.

631 He S, Ivanova N, Kirton E, Allgaier M, Bergin C, Scheffrahn RH, Kyrpides NC, Nikos C,

632 Warnecke F, Tringe SG, Hugenholtz F. 2013. Comparative Metagenomic and

633 Metatranscriptomic Analysis of Hindgut Paunch Microbiota in Wood- and Dung-Feeding Higher

634 Termites. *PLoS ONE* 8: e61126. DOI: 10.1371/journal.pone.0061126.

635 Hoback WW, Stanley DW. 2001. Insects in hypoxia. *Journal of Insect Physiology* 47: 533–542.

636 DOI: 10.1016/s0022-1910(00)00153-0.

637 Hongoh Y. 2010. Diversity and Genomes of Uncultured Microbial Symbionts in the Termite

638 Gut. *Bioscience, Biotechnology and Biochemistry* 74: 1145–1151. DOI: 10.1271/bbb.100094.

639 Hongoh Y, Deevong P, Inoue T, Moriya S, Trakulnaleamsai S, Ohkuma M. 2005. Intra- and

640 interspecific comparisons of bacterial diversity and community structure support coevolution of

641 gut microbiota and termite host. *Applied and Environmental Microbiology* 71(11): 6590–6599.

642   DOI: 10.1128/AEM.71.11.6590-6599.2005.Huse SM, Ye Y, Zhou Y, Fodor AA. 2012. A core

643   human microbiome as viewed through 16S rRNA sequence clusters. *PLoS ONE* 7: e34242. DOI:

644   10.1371/journal.pone.0034242.

645   Jones DT, Eggleton P. Global biogeography of termites. In: Bignell DE, Roisin Y, Lo N. (eds).

646   2011. *Biology of Termites: A Modern Synthesis*. Dordrecht: Springer, 2011, 477-98.

647   Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple

648   sequence alignment based on fast Fourier transform. *Nucleic Acids Research*. 30: 3059–3066.

649   DOI: 10.1093/nar/gkf436.

650   Köhler T, Dietrich C, Scheffrahn RH, Brune A. 2012. High-Resolution Analysis of Gut

651   Environment and Bacterial Microbiota Reveals Functional Compartmentation of the Gut in

652   Wood-Feeding Higher Termites (*Nasutitermes* spp.). *Applied and Environmental Microbiology*

653   78: 4691–4701. DOI: 10.1128/AEM.00683-12.

654   Krishna K, Grimaldi DA, Krishna V, Engel MS. 2013. Treatise on the Isoptera of the world.

655   *Bulletin of the American Museum of Natural History* 377: 2704.

656   Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA. 2001. Nitrogen

657   fixation by symbiotic and free-living Spirochetes. *Science* 292: 2495–2498. DOI:

658   10.1126/science.1060281.

659   Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. 2011. UniFrac: an effective

660   distance metric for microbial community comparison. *The ISME Journal* 5: 169–172. DOI:

661   10.1038/ismej.2010.133.

662   McMurdie PJ, Holmes S. 2013. Phyloseq: An R Package for Reproducible Interactive Analysis

663   and Graphics of Microbiome Census Data. *PLOS ONE* 8(4): e61217. DOI:

664   10.1371/journal.pone.0061217.

665 Marynowska M, Goux X, Sillam-Dussès D, Rouland-Lefèvre C, Halder R, Wilmes P, Gawron P,

666 Roisin Y, Delfosse P, Calusinska M. 2020. Compositional and functional characterisation of

667 biomass-degrading microbial communities in guts of plant fibre- and soil-feeding higher

668 termites. *Microbiome* 8(1): 96. DOI: 10.1186/s40168-020-00872-3.

669 Mikaelyan A, Dietrich C, Köhler T, Poulsen M, Sillam-Dussès D, Brune A. 2015. Diet is the

670 primary determinant of bacterial communitystructure in the guts of higher termites. *Molecular*

671 *Ecology* 24: 5284–5295. DOI: 10.1111/mec.13376.

672 Mikaelyan A, Meuser K, Brune A. 2017. Microenvironmental heterogeneity of gut

673 compartments drives bacterial community structure in wood- and humus-feeding higher termites.

674 *FEMS Microbiology Ecology* 93(1):fiw210. DOI: 10.1093/femsec/fiw210.

675 Morewood WD, Hoover K, Neiner PR, McNeil JR, Sellmer JC. 2004. Host tree resistance

676 against the polyphagouswood-boring beetle *Anoplophora glabripenn. Entomologia*

677 *Experimentalis et Applicata* 110(1): 79–86. DOI: 10.1111/j.0013-8703.2004.00120.x.

678 Morgan JL, Darling AE, Eisen JA. 2010. Metagenomic sequencing of an in vitro-simulated

679 microbial community. *PLoS ONE* 5(4):e10209. DOI: 10.1371/journal.pone.0010209.

680 Ni J, Tokuda G. 2013. Lignocellulose-degrading enzymes from termites and their symbiotic

681 microbiota. *Biotechnology Advances* 31: 838–850. DOI: 10.1016/j.biotechadv.2013.04.005.

682 Otani S, Mikaelyan A, Nobre T, Hansen LH, Koné NA, Sørensen SJ, Aanen DK, Boomsma JJ,

683 Brune A, Poulsen M. 2014. Identifying the core microbial community in the gut of fungus-

684 growing termites. *Molecular Ecology* 23(18): 4631–4644. DOI: 10.1111/mec.12874.

685 Paul K, Nonoh JO, Mikulski L, Brune A. 2012. 'Methanoplasmatales': Thermoplasmatales-

686 related archaea in termite guts and other environments are the seventh order of methanogens.

687 *Applied and Environmental Microbiology* 78: 8245–8253. DOI: 10.1128/AEM.02193-12.

688   Paulino LC, Tseng CH, Blaser MJ. 2008. Analysis of *Malassezia* microbiota in healthy

689   superficial human skin and in psoriatic lesions by multiplex real-time PCR. *FEMS Yeast*

690   *Research* 8: 460–471. DOI: 10.1111/j.1567-1364.2008.00359.x.

691   Price MN, Dehal PS, Arkin AP. 2010. FastTree 2--approximately maximum-likelihood trees for

692   large alignments. *PLoS ONE* 5: e9490. DOI:10.1371/journal.pone.0009490.

693   Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The

694   SILVA ribosomal RNA gene database project: improved data processing and web-based tools.

695   *Nucleic Acids Research* 41: 590–596. DOI: 10.1093/nar/gks1219.

696   Rahman NA, Parks DH, Willner DL, Engelbrektson AL, Goffredi SK, Warnecke F, Scheffrahn

697   RH, Hugenholtz P. 2015. A molecular survey of Australian and North American termite genera

698   indicates that vertical inheritance is the primary force shaping termite gut microbiomes.

699   *Microbiome* 3: 2–16. DOI: 10.1186/s40168-015-0067-8.

700   Romero Victorica M, Soria MA, Batista-García RA, Ceja-Navarro JA, Vikram S, OrtizM,

701   Ontañon O, Ghio S,Martínez-Ávila L, Quintero García OJ, Etcheverry C, Campos E, Cowan D,

702   Arneodo J, Talia PM. 2020. Neotropical termite microbiomes as sources of novel plant cell wall

703   degrading enzymes. *Scientific Reports* 10(3864). DOI: 10.1038/s41598-020-60850-5.

704   Santana RH, Catão EC, Lopes FA, Constantino R, Barreto CC, Krüger RH. 2015. The gut

705   microbiota of workers of the litter-feeding termite *Syntermes wheeleri* (Termitidae:

706   Syntermitinae): archaeal, bacterial, and fungal communities. *Microbial Ecology* 70: 545–556.

707   DOI: 10.1007/s00248-015-0581-z.

708   Scheffrahn RH, Krecek J, Szalanski AL, Austin JW. 2005. Synonymy of Neotropical Arboreasl

709   Termites *Nasutitermes corniger* and *N. costalis* (Isoptera: Termitidae: Nasutermitinae), with

710 Evidence from Morphology, Genetics, and Biogeography. *Annals of the Entomological Society*

711 *of America* 98: 273–281. DOI:10.1603/0013-8746(2005)098[0273:SONATN]2.0.CO;2.

712 Shade A, Handelsman J. 2012. Beyond the Venn diagram: the hunt for a core microbiome.

713 *Environmental Microbiology* 14: 4–12. DOI: 10.1111/j.1462-2920.2011.02585.x.

714 Shi W, Xie S, Chen X, Sun S, Zhou X, Liu L, Gao P, Kyrpides NC, No EG, Yuan*et* JS. 2013.

715 Comparative Genomic Analysis of the Microbiome of Herbivorous Insects Reveals Eco-

716 Environmental Adaptations: Biotechnology Applications. *PLoS Genetics* 9: 1-12. DOI:

717 10.1371/journal.pgen.100313.

718 Souza G, Dos Santos VC, de Figueirredo Gontijo N, Constantino R, de Oliveira Paiva E, Silva

719 G, Bahia AC, Mendonça Gomes F, de Alcantara Machado E. 2017. Morphophysiological study

720 of digestive system litter-feeding termite *Cornitermes cumulans* (Kollar, 1832). *Cell and Tissue*

721 *Research* 368: 579–590. DOI: 10.1007/s00441-017-2584-1.

722 Su L, Yang L, Huang S, Su X, Li Y, Wang FQ, Wang E, Kang N, Xu J, Song A. 2016.

723 Comparative Gut Microbiomes of Four Species Representing the Higher and the Lower

724 Termites. *Journal of Insect Science* 16: 1–9. DOI: 10.1093/jisesa/iew081.

725 Tai V, James ER, Nalepa CA, Scheffrahn RH, Perlman SJ, Keeling PJ. 2015. The role of host

726 phylogeny varies in shaping microbial diversity in the hindguts of lower termites. *Applied and*

727 *Environmental Microbiology* 81: 1059–1070. DOI: 10.1128/AEM.02945-14.

728 Turner S, Pryer KM, Miao VP, Palmer JD.1999. Investigating deep phylogenetic relationships

729 among cyanobacteria and plastids by smalls ubunit rRNAsequence analysis. *Journal of Eukaryot*

730 *Microbiology* 46, 327–338. DOI: 10.1111/j. 1550-7408.1999.tb04612.x.

731 Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M,

732 Mchardy AC, Djordjevic G, Aboushadi N, Sorek R, Tringe SG, Podar M, Martin HG, Kunin V,

733 Dalevi D, Madejska J, Kirton E, Platt D, Szeto E, Salamov A, Barry K, Mikhailova N, Kyrpides

734 NC, Matson EG, Ottesen EA, Zhang X, Hernández M, Murillo C, Acosta LG, Rigoutsos I,

735 Tamayo G, Green BD, Chang C, Rubin EM, Mathur EJ, Robertson DE, Hugenholtz P,

736 Leadbetter JR. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-

737 feeding higher termite. *Nature* 450: 560–565. DOI: 10.1038/nature06269.

738 Yamada A, Inoue T, Noda Y, Hongoh H, Ohkuma M. 2007. Evolutionary trend of phylogenetic

739 diversity of nitrogen fixation genes in the gut community of wood-feeding termites. *Molecular*

740 *Ecology* 16: 3768–3777. DOI: 10.1111/j.1365-294X.2007.03326.x.

741 Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, Yoon C, Nam YD, Kim YJ, Choi JH,

742 Kim JY, Shin NR, Kim SH, Lee WJ, Bae JW. 2014. Insect gut bacterial diversity determined by

743 environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and*

744 *Environmental Microbiology* 80: 5254-64. DOI: 10.1128/AEM.01226-14.

745 Zhang N, Su SO, Blackwell M. 2003. Microorganisms in the gut of beetles: evidence from

746 molecular cloning. *Journal of Invertebrate Pathology* 84: 226–233. DOI:

747 10.1016/j.jip.2003.10.002.

748 Zhang Z, Jiao S, Li X, and Li M. 2018. Bacterial and fungal gut communities of *Agrilus mali* at

749 different developmental stages and fed different diets. *Scientific Reports* 8: 15634. DOI:

750 10.1038/s41598-018-34127-x.

Figure 1

Relative abundance of bacteria, archaea (phyla level) and fungi (class level) in the gut of Neotropical termites.

(A) 16S rRNA gene (B) ITS sequence based taxonomic distribution in triplicate gut samples.

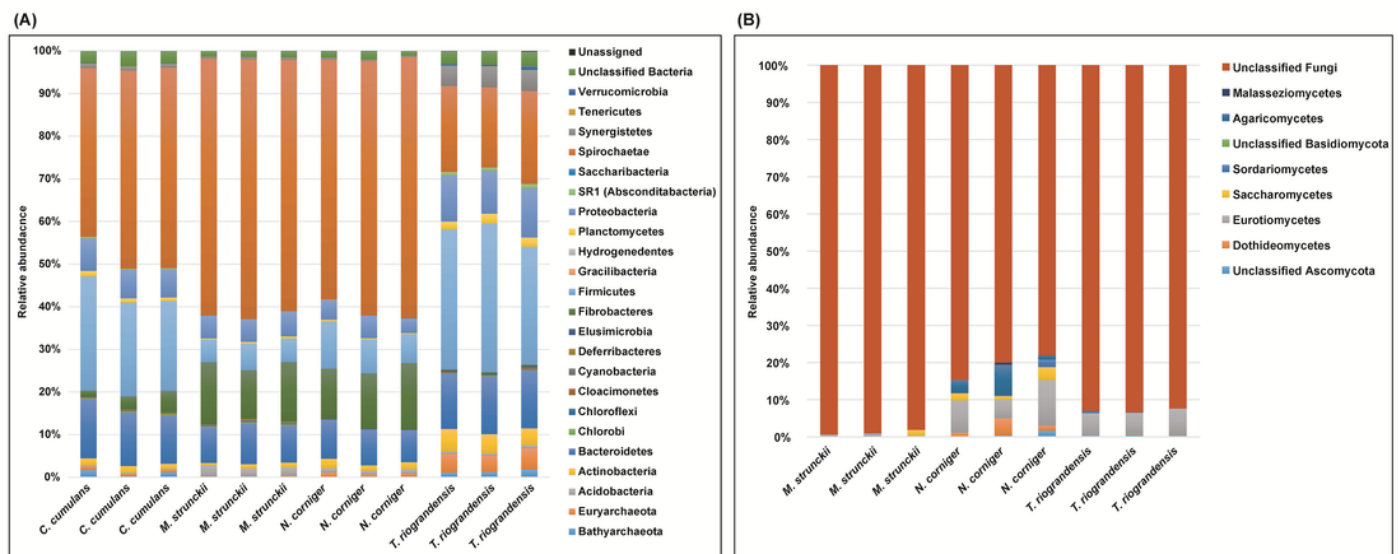



Figure 2

Rarefaction curves for the prokaryotic and fungi observed ASVs at 97% sequence identity.

(A) 16S rRNA gene sequences of *C. cumulans*, *M. strunckii*, *N. corniger* and *T. riograndensis*.

(B) ITS sequences. (C) 16S rRNA gene of the total ASVs and sequence counts showing the sufficient coverage at the 14,042 sequences per sample.

(A)

(B)

(C)

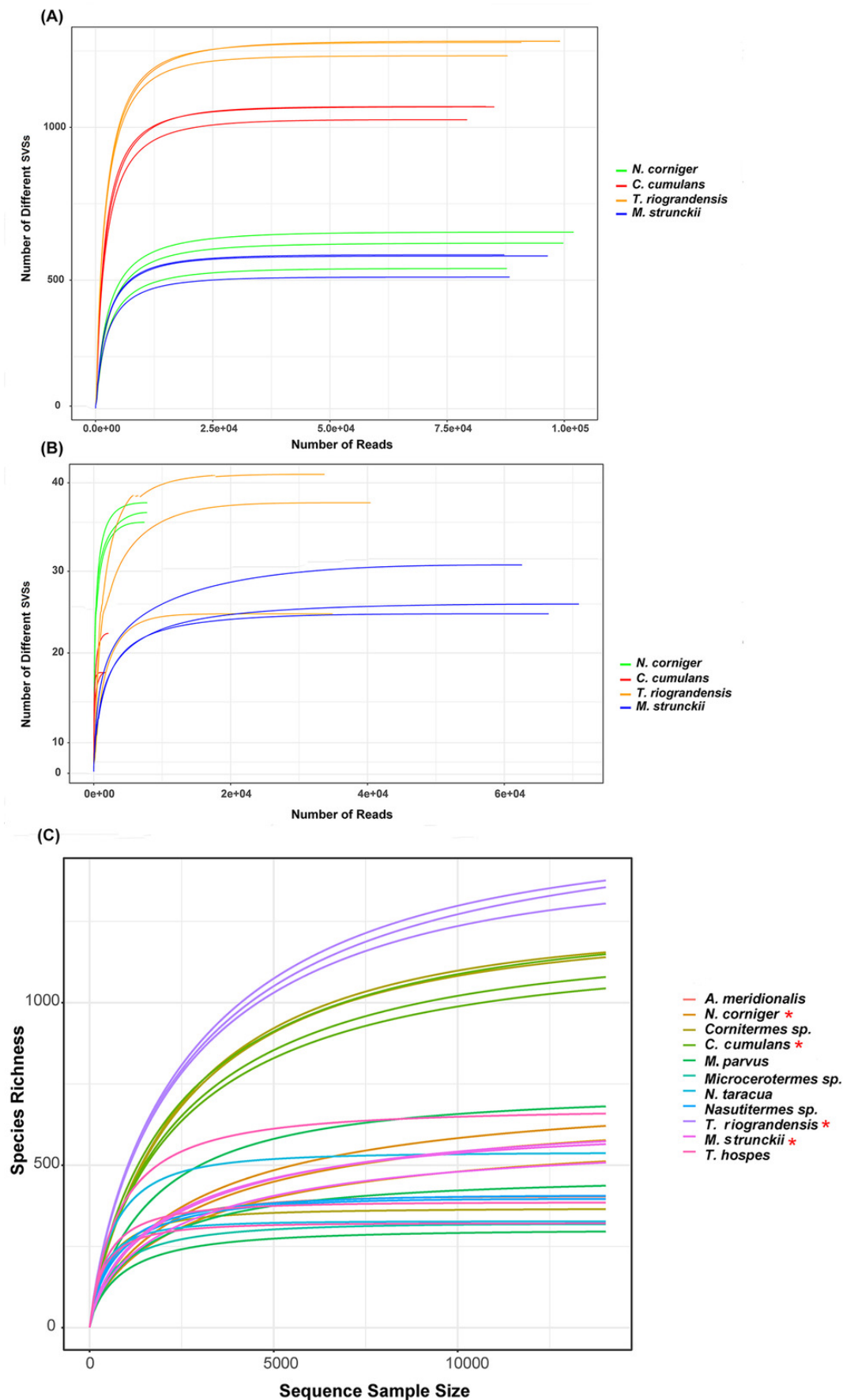



Figure 3

Prokaryotic α-diversity measures.

(A) Shannon, (B) Pielou's evenness and (C) number of observed ASVs of the four species of

termites. Comparisons were performed using the Kruskal-Wallis followed by *Benjamini &*

Hochberg FDR correction.

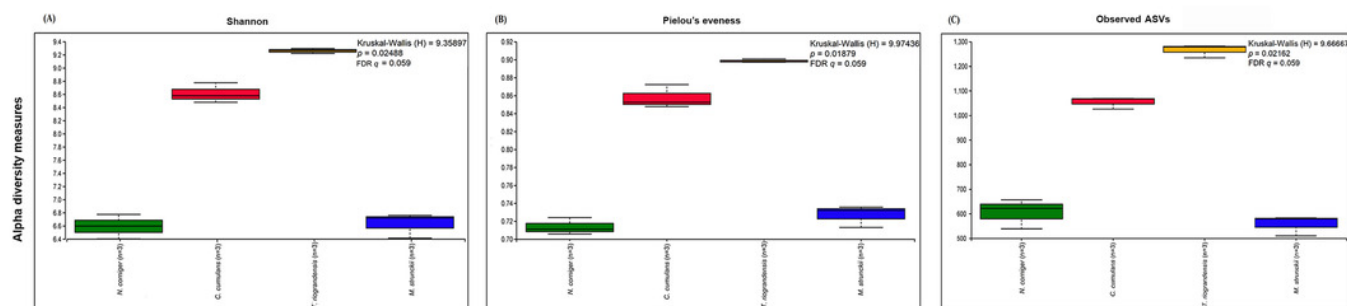



Figure 4

Prokaryotic α-diversity measures.

(A) Shannon, (B) Pielou's e venness and (C) number of observed ASVs of the total species of higher termites used to compare between diet groups. Comparisons were performed using the Kruskal-Wallis followed by *Benjamini & Hochberg* FDR correction . The H stats, *p* value and corrected *p* value (*q*) is written on the top of the paired comparison between the diet groups.

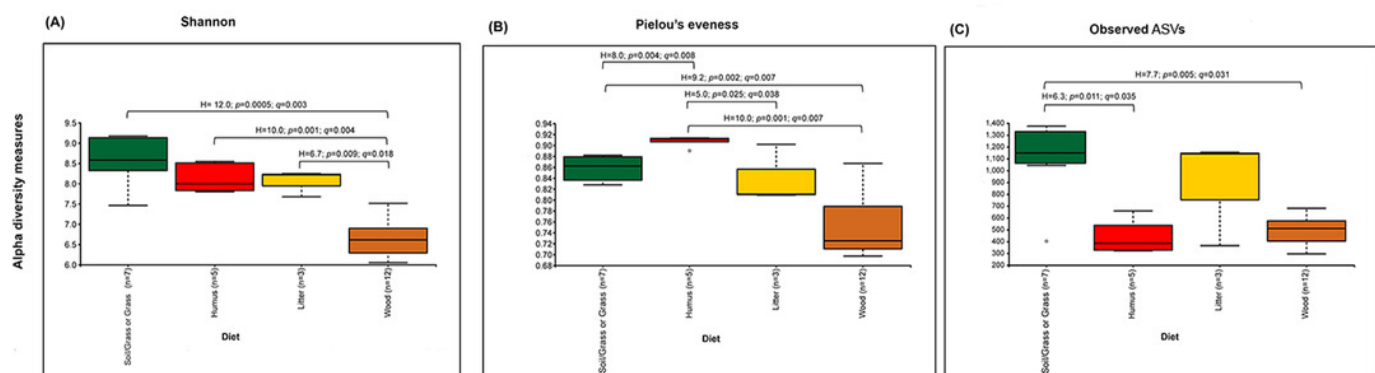



Figure 5

Beta-diversity NMDS plot of Unifrac distances.

(A) Unweighted Unifrac distance plot. The red, blue, green and orange dots represent the gut microbiome of *C. cumulans*, *M. strunckii*, *N. corniger* and *T. riograndensis*, respectively. The circle and triangle denote the soil/grass and wood feeders, respectively. The first and second principal components explain 34% (PC1) and 24% (PC2) of variations, respectively. (B) Unweighted unifrac distance of a larger number of host species (C) Weighted Unifrac distance of a larger number of host species.

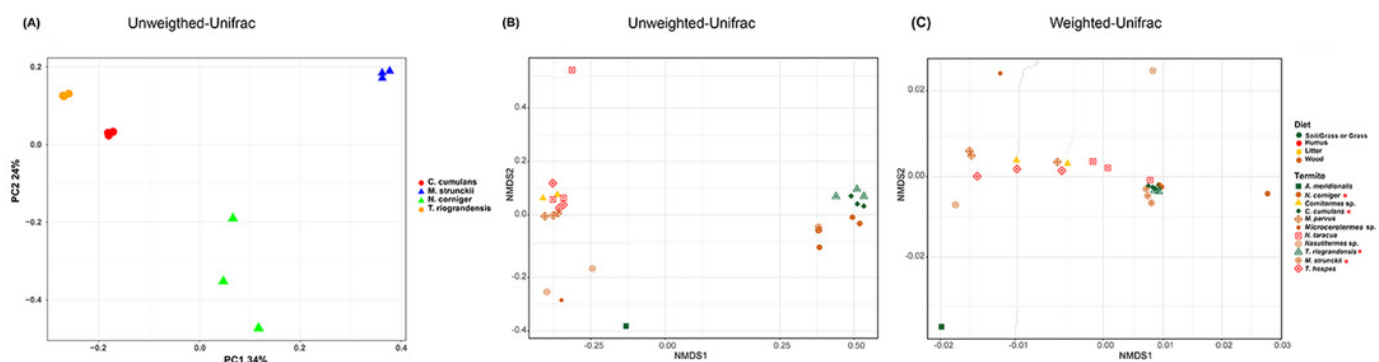
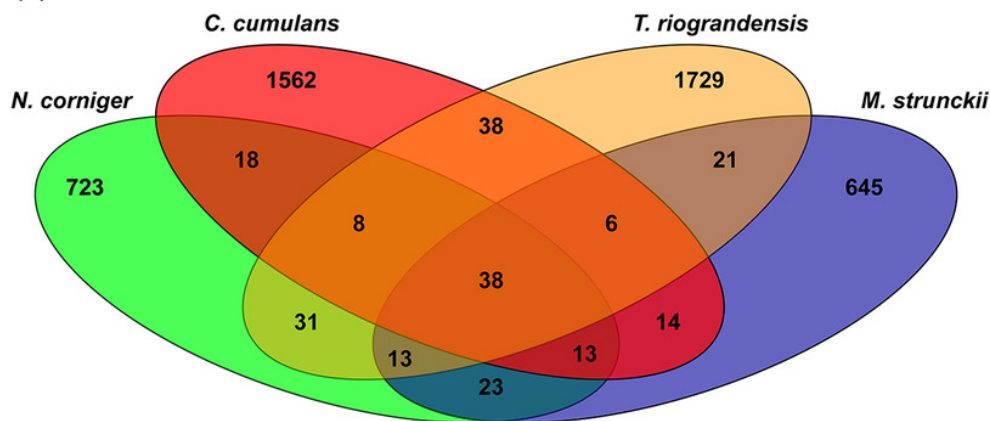
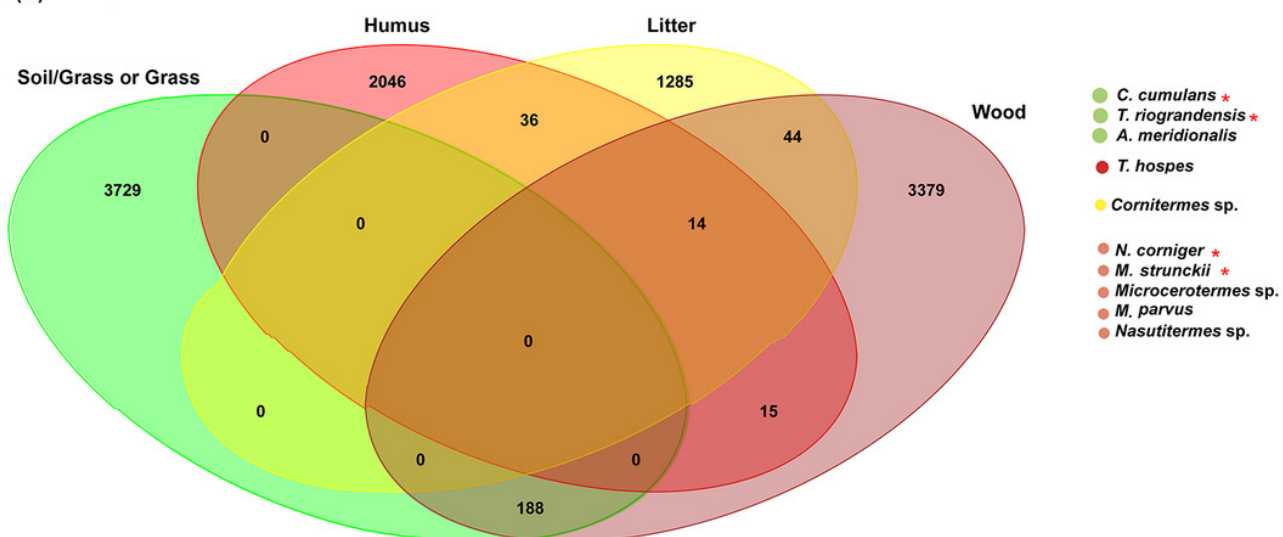
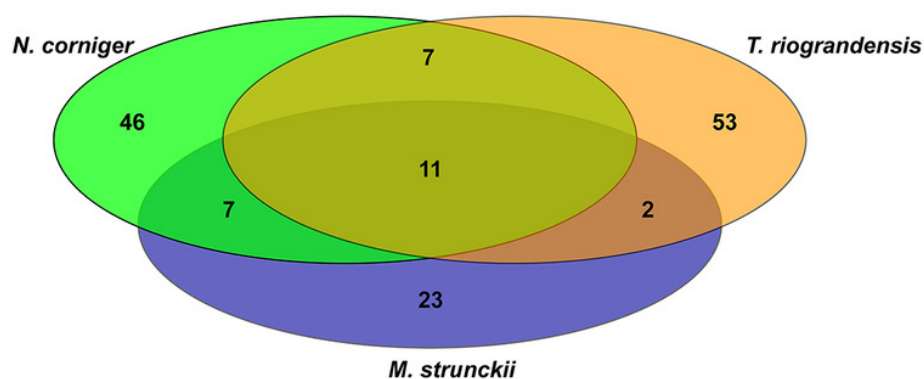



Figure 6

Venn diagram showing the distribution of shared ASVs across the termite gut.

(A) Shared prokaryotic ASVs between four gut microbiomes (B) Shared prokaryotic ASVs between four gut microbiomes (B) Shared prokaryotic ASVs between diet groups of termites and (C) Shared fungal ASVs between the three termites.

(A)

(B)

(C)

Summary of the higher termites used in the current study, their taxonomy classification, feeding groups and accession numbers of bacterial 16S rRNA amplicon libraries.

*Termites collected in this study.

[a]Based on the food types given for termite genera (*Jones & Eggleton, 2011*).

[b]Based on the dietary information in *Souza et al.,* (*2017*).

[c]Based on dietary information in *French & Ahmed* (*2011*).

[d]Based on observations of *Gontijo & Domingos* (*1991*).

Host species	Subfamily	Diet preferences	Replicates	NCBI Biosample ID	References
*Nasutitermes corniger**	Nasutitermitinae	Wood[a]	(n=3)	SAMN09635494	This work
Nasutitermes sp.	Nasutitermitinae	Wood[a]	(n=2)	SAMN08180495	*Bourguignonet al., 2018*
Microcerotermes sp. E	Termitinae	Wood[a]	(n=1)	SAMN08180514	*Bourguignon et al., 2018*
*Microcerotermes strunckii**	Termitinae	Wood[a]	(n=3)	SAMN09635494	This work
Microcerotermes parvus Mp193	Termitinae	Wood[a]	(n=3)	SAMN04317068–70	*Mykaelyan et al., 2017*
*Cornitermes cumulans**	Syntermitinae	Grass[b]	(n=3)	SAMN09635494	This work
Amitermes meridionalis	Termitinae	Grass[c]	(n=1)	SAMN08180513	*Bourguignon et al., 2018*
*Termes riograndensis**	Termitinae	Soil/Grass	(n=3)	SAMN09635494	This work
Neocapritermes taracua Nt197	Termitinae	Humus[a]	(n=2)	SAMN04317074–76	*Mykaelyan et al., 2017*
Termes hospes Th196	Termitinae	Humus[a]	(n=3)	SAMN04317083–85	*Mykaelyan et al., 2017*
Cornitermes sp. Co191	Syntermitinae	Litter[a]	(n=3)	SAMN04317065-67	*Mykaelyan et al., 2017*

PERMANOVA analysis of bacteria/archaea and fungi.

The statistical analysis of ITS sequence data were performed based on the Hellinger transformation and Bray-Curtis distance-based dissimilarity matrix.

16S rRNA				
	Based on four termite species		**Based on 11 termite species**	
	Unweighted Unifrac	Weighted Unifrac	Unweighted Unifrac	Weighted Unifrac
Pseudo F	8.5445	67.0377	1.5075	1.5407
***p* value**	0.001	0.001	0.031	0.199
	Based on diet groups (Grass and Wood)		**Based on diet groups (Soil/Grass or Grass, Humus, Litter, and Wood)**	
Pseudo F	4.5241	12.4488	3.74797	5.37108
***p* value**	0.002	0.002	0.001	0.005
ITS (Bray-Curtis distance dissimilarity matrix)				
	Based on four termite species			
Pseudo F	13.1240			
***p* value**	0.005			
	Based on diet groups (Grass and Wood)			
Pseudo F	3.8839			
***p* value**	0.03			