1   **Leprous lesion reveals disturbed skin-resident microbiota**

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15   Keywords: Leprosy; 16S rRNA gene; skin; diversity; microbiota

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ABSTRACT

Leprosy is a chronic infectious disease that remains a major challenge to public health in endemic countries. Increasing evidence has highlighted the importance of microbiota for human general health and, as such, the study of skin microbiota is of interest. But while studies are continuously revealing the complexity of human skin microbiota, the microbiota of leprous cutaneous lesions has not yet been characterized. Here we used Sanger and massively parallel SSU rRNA gene sequencing to characterize the microbiota of leprous lesions, and studied how it differs from the bacterial skin composition of healthy individuals previously described in the literature. Taxonomic analysis of leprous lesions revealed main four phyla: Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, with Proteobacteria presenting the highest diversity. There were considerable differences in the distribution of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria, with the first two phyla enriched and the other markedly diminished in the leprous lesions, when compared with healthy skin. *Propionibacterium*, *Corynebacterium* and *Staphylococcus*, resident and abundant in healthy skin, were underrepresented in skin from leprous lesions. Most of the taxa found in skin from leprous lesions are not typical of human skin and potentially pathogenic, with the *Bulkorderia*, *Pseudomonas* and *Bacillus* genera being overrepresented. Our data suggest significant shifts of the microbiota with emergence and competitive advantage of potentially pathogenic bacteria over skin resident taxa.

INTRODUCTION

Mycobacterium leprae is the causative agent of leprosy, an ancient chronic infectious disease and may have severely debilitating physical, social, and psychological consequences. The skin, the peripheral nerves, the nasal mucosa, eyes, and the reticulum-endothelial system are the preferred target sites for this pathogen. The disease displays a spectrum of clinical manifestations, such as lepromatous (multibacillar) and tuberculoid (paucibacillar) leprosy, which are attributed to the host immune response. It still remains a stigmatizing disease (*Nascimento, 2013; Degang et al., 2014*). This neglected tropical disease has a close relationship with poverty, being a major challenge to public health in countries where it remains endemic. Data reported by the World Health Organization in 2013 revealed that, in 2012, around 122 countries presented cases of leprosy with India showing the highest number of cases (134,752) followed by Brazil (33,303).

Increasing evidence is continuously bringing to light the importance of microbiota for human general health, including its essential role in physiology, and in our immune responses and metabolism (*Cho & Blaser, 2012*). Thus, the human microbiome has been referred to as a forgotten organ (*Morgan & Huttenhower, 2012*). New sequencing technologies are transforming the study of microbial diversity and have revealed that the human skin harbors a complex microbiota. Previous studies highlight that the human skin microbiome is diverse and personalized (*Costello et al., 2009; Grice et al., 2009*). Indeed, among the 19 bacterial phyla found so far by these studies, special attention goes to the Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes phyla, which are consistently reported and account for 99% of the 16S

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75 rRNA gene sequences. These studies have also uncovered the genera *Corynebacterium*,

76 *Propionibacterium*, and *Staphylococcus* as abundant resident microbiota of human skin.

77 Other microbiome studies have provided insights into the delicate balance

78 between skin health and disease (*Gao et al., 2008; Costello et al., 2009; Grice et al.,*

79 *2009; Kong et al., 2012*). Studies on the skin microbiota of individuals with non-

80 infectious diseases, such as atopic dermatitis and psoriasis, have revealed a variation in

81 the bacterial composition of the skin of these patients when compared to healthy

82 persons (*Dekio et al., 2007; Gao et al., 2008; Kong et al., 2012*). In comparison to

83 healthy individuals, atopic dermatitis patients show a greater abundance of

84 *Stenotrophomonas maltophilia*, and a lower abundance of *Propionibacterium acnes* and

85 *Staphylococcus* sp., both resident skin bacteria (*Dekio et al., 2007*). In patients with

86 psoriatic lesions, the most abundant phylum was Firmicutes and least abundant

87 Actinobacteria (*Gao et al., 2008*). However, studies on the bacterial community

88 composition of the skin of individuals with leprosy are still missing.

89 In this study we characterized the skin microbiota of leprous lesions to

90 determine whether it differs from the skin bacterial composition of healthy individuals

91 by sequencing a 16S rRNA clone library. The data presented herein have important

92 implications to foster research about the role of skin microbiota in leprosy.

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94 **METHODS**

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96 **Ethics statement**

97 The study was approved by the Universidade Federal de Minas Gerais Research

98 Ethical Committee with approval number CAAE - 0709.0.203.000-11. The leprous skin

99 samples were obtained from Hermes Pardini pathological anatomy laboratory of Belo

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100　Horizonte, Brazil. The samples were rendered anonymized for researchers before its

101　use.

102

Specimen and DNA extraction

104　　　Samples studied were archival formalin-fixed paraffin embedded sections of

105　lepromatous leprosy lesion skin. The skin biopsies measuring approximately 3 x 3 mm

106　were collected from nare and volar forearm prior to antimycobacterial treatment. Before

107　proceeding to the DNA extraction the paraffin blocks were washed with ethanol 70%,

108　for decontamination, and a new blade was placed in the microtome. The first sections

109　were discarded and the next ones were used for DNA extraction. DNA extraction was

110　carried out according to a procedure modified from *Coura, Prolla & Ashton-Prolla et*

111　*al., (2005)*. After the procedure of digestion with proteinase K, DNA extraction was

112　continued using phenol-chloroform as described by *Sambrook et al.* (*1989*). Total DNA

113　was quantified by absorbance at 260 nm using a NanoDrop Spectrophotometer

114　(NanoDrop Technologies). DNA purity was assessed using the A260/A280 ratio. The

115　DNA was stored at -20 °C until further processing. We also included in the analysis the

116　results from samples previously obtained from psoriasis and atopic dermatitis patients

117　and from healthy persons (*Dekio et al., 2007; Gao et al., 2008; Costello et al., 2009;*

118　*Grice et al., 2009; Kong et al., 2012*).

119

PCR amplification of the 16S rRNA gene, cloning and Sanger sequencing

121　　　The bacterial 16S rRNA gene fragment was amplified using touchdown PCR

122　according to *Freitas et al.* (*2008*), with the conserved primer set 8f (5'-

123　AGAGTTTGATCMTGGCTCAG-3') and 907r (5'-

124　TACGGHTACCTTGTTACGACTT3-') (*Lane, 1991*). The amplicons were gel-

125 purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany), cloned into

126 the vector pJET1.2/blunt (Fermentas, Canada) according to the manufacturer's

127 instructions, and transformed into electrocompetent *Escherichia coli* DH5α. The 16S

128 rDNA fragments were sequenced bidirectionally using the pJET1.2 forward and reverse

129 primers and an ABI Prism 3130 DNA sequencer (Applied Biosystems, Foster City,

130 CA).

131

132 **Phylogenetic analysis**

133 Sequences were assembled using Linux programs Phred/Phrap/Consed

134 (http://www.phrap.org/phredphrapconsed.html). Chimeric sequences were identified

135 using Bellerophon (*Huber, Faulkner & Hugenholtz, 2004*). Good's coverage (*Good,*

136 *1953*) and rarefaction curves were calculated for operational taxonomic units (OTUs)

137 with an evolutionary distance of 0.03, using DOTUR program (*Schloss & Handelsman,*

138 *2005*). The OTUs were compared with available databases using the BLASTn search

139 tool from GenBank (http://www.ncbi.nlm.nih.gov/). Sequence alignment and

140 phylogenetic relationships were inferred with ARB (*Ludwig, et al., 2004; Pruesse, et*

141 *al., 2007*) using the neighbor-joining algorithm (http://www.arb-home.de). The

142 bootstrap consensus tree inferred from 500 replicates (*Felsenstein, 1985*)] was taken to

143 represent the evolutionary history of the taxa analyzed. The nucleotide sequences

144 generated were deposited in the GenBank database under the accession numbers KJ

145 022641 to KJ 022699.

146

147 **V3-V4 hypervariable regions PCR amplification and massively parallel sequencing**

148   Amplification of the V3-V4 hypervariable regions was performed using the

149 region-specific bacterial/archaeal primers S-D-Bact-0341-b-S-17 forward 5'-

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150 CCTACGGGNGGCWGCAG-3'   and   S-D-Bact-0785-a-A-21   reverse   5'-

151 GACTACHVGGGTATCTAATCC-3' (*Kozich, et al., 2013*), with Illumina adapters

152 added. Barcoded amplicons were generated using KAPA HiFi HotStart ReadyMix

153 (KAPA, Woburn, MA, USA) and purified using AMPure XP beads (Agencourt

154 Bioscience, Beverley, MA, USA). Sequencing was performed using the MiSeq platform

155 (Illumina, Inc., San Diego, CA, USA) according to manufacturer's instructions.

156
157 **Bioinformatics analysis**

158 16S rRNA microbiota primary data analysis was performed with PRINSEQ (stand alone

159 lite version, http://prinseq.sourceforge.net/) where quality-based trimming was done.

160 Reads with N's or an overall mean Q-score < 25 were discarded. The resulting fasta file

161 was also screened for ambiguous base and homopolymers by using MOTHUR v.1.33.0

162 (http://www.mothur.org). Chimeras were detected using the UCHIME algorithm

163 (http://drive5.com/uchime). Moreover, OTUs and taxonomic classification were

164 determined using the closed-reference strategy implemented in QIIME 1.8 (*Caporaso,*

165 *et al., 2010*), with reads clustered at 97% of similarity, against the Greengenes reference

166 database (from August 2013). The nucleotide sequences were submitted to Sequence

167 Read Archive (SRA) with the accession number of XX.

168
169 **RESULTS**

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171   The bacterial composition of leprous lesions was investigated using traditional

172 and massively parallel sequencing. We first studied the bacterial community by Sanger

173 sequencing constructing a 16S rRNA gene library of one skin biopsy sample.

174 Rarefaction analysis indicated that diversity was reasonably well sampled, as evidenced

175 by the nonasymptotic curve presented in Fig. 1, a result concordant with the Good's

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coverage data (73%). A total of 88 clones were randomly picked and sequenced. Fifty-nine 16S rRNA gene sequences were obtained after quality control and removal of chimeric sequences. The partial 16S rRNA gene sequences used for phylogenetic analysis were 600 nucleotides long and spanned the V2 to V5 hypervariable regions corresponding to *Escherichia coli* K12.

To determine the bacterial diversity associated with leprosy, the 16S rDNA clone sequences were analyzed phylogenetically. They were distributed into 27 OTUs spanning four bacterial phyla. The relative abundance of the phylogenetic groups as well as the resulting phylogenetic tree are shown in Figs. 2 and 3, respectively.

The largest fractions of the clone library were represented by Proteobacteria (48%) and Firmicutes (41%). Actinobacteria, the most prevalent and diverse phylum in normal skin from healthy persons, was underrepresented in the leprous sample analyzed. Bacteroidetes phylum comprised the smallest fraction (Fig. 2).

Proteobacteria was characterized by a broad diversity with the most abundant OTU classified at genus level as *Burkholderia*, and the other OTUs as *Klebsiella*, *Hydrogenophilus*, *Pseudomonas*, *Achromobacter*, *Sphingomonas*, and *Rhodoplanes* were evenly abundant (3.7% each). In contrast, Bacteroidetes was represented by a single genus, *Dyadoabacter*. The most abundant Firmicutes OTU was of the *Bacillus* genus (14.8%), whereas *Propionibacterium* and *Staphylococcus*, typical resident bacteria of normal skin, were less abundant (3.7% each) (Fig. 2). The order *Actinomycetales*, which harbors the species *Mycobacterium leprae*, was represented in our study by the genus *Nocardioides* (Fig. 2).

Most OTUs displayed relationships with sequences of culturable bacteria obtained from a wide range of environments, from volcanic to copper mining. Only two OTUs were related to culturable bacteria from human body sites, skin and vagina.

Furthermore, eight OTUs for which no corresponding cultured genera are known, included sequences most similar to the class Gammaproteobacteria (1 OTU), order Bacillales (1 OTU) and families Bacillaceae (2 OTUs), Planococcaceae (1 OTU), Methylocystaceae (1 OTU), and Xanthomonadaceae (2 OTUs), and thus may represent novel bacterial taxa (Table S1).

To reveal the fine details of leprous lesions microbiota we conducted massively parallel sequencing on the V3-V4 hypervariable regions of the 16S rRNA gene (abbreviated henceforth as V3-V4 tag). V3-V4 tag of two skin biopsy samples yielded a total of 80 514 high quality reads (17 038 of S1 and 63 476 of S2), with the average read length of 455 bp. The Good's coverage values (99.2% and 99.8%) and rarefaction curves (Fig. 1) obtained with an evolutionary distance of 0.03 indicated that the diversity was thoroughly uncovered. The reads were clustered into 1 084 OTUs (562 of S1 and 522 of S2), spanning a total of 27 phyla (Fig. 4). Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes represented 88.3% of all reads. The main four phyla were the unique found in the Sanger sequencing. The minor bacterial phyla were Acidobacteria, Chloroflexi and Nitrosprae, accounting for 5.5% of all reads. The group "other bacteria" comprised Gemmatimonadetes, Cyanobacteria, Verrucomicrobia, OP3, GN04 Elusimicrobia, Planctomycetes, Fusobacteria, among others, representing 10.7% of the OTUs.

The most abundant phylum was Proteobacteria, which comprised more than half of all reads. Reads affiliated with Gamma- and Alphaproteobacteria predominated, constituting 72.5% of all proteobacterial reads. The remaining reads belonged to Beta- (22.1%), Delta (5.4%) and Epsilonproteobacteria (0.0001%). As in the Sanger sequencing, Proteobacteria harbored wide diversity, totalizing 50 families and 79 genera. Among the 10 top proteobacterial taxa there were representatives from different families or genera,

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226   namely, *Pseudomonas* (32.4%), *Sphingomonas* (13.7%), Caulobacteraceae (15%),

227   Xanthomonadaceae (5.3%), Alcaligenaceae (2.5%), *Proteus* (1.7%), *Gallionella* (3.9%),

228   Comamonadaceae (9.8%), *Chromobacterium* (1.3%) and *Crenothrix* (2.5%), accounting

229   for 88.1% of all proteobacterial reads.

230   In contrast to Sanger sequencing, Bacteroidetes was the second most abundant

231   phylum. Seventy-one percent of all Bacteroidetes-associated reads were affiliated with

232   the Flavobacteriaceae family. Other taxa found were *Sphingobacterium*, *Leadbetterella*,

233   and *Elizabethkingia meningoseptica*.

234   Streptococcaceae, Planococcaceae, Bacillaceae, Ruminococcaceae and

235   Staphylococcaceae were the members dominants of Firmicutes. The genus

236   *Streptococcus* comprised almost half of all Firmicutes-associated reads, whereas

237   representation of the genus *Sataphylococcus* was much low (0.2%).

238   Actinobaceria were underrepresented in the samples, in agreement with the

239   Sanger sequencing. Within of Actinobacteria, the Micrococaceae and

240   Intrasporangiaceae families were the most common and contained 36.6% and 16.6%.

241   Nevertheless, *Propionibacterium* (0.7%) and *Corynebacterium* (0.4%) were also found

242   in less abundance. It should be noted that *Mycobacterium* were represented by a few

243   reads (0.0007%).

244

245   **DISCUSSION**

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247   Leprosy is a stigmatizing disease because of the deformation caused by the skin

248   lesions displayed by infected individuals. Recent investigations have highlighted the

249   role of skin microbiota at the interface of health and disease (*Cho & Blaser, 2012*).

250   Thus, accurate characterization of skin bacterial communities is an important challenge

251 in the search for possible links between microbiota changes and disease. The current

252 study used Sanger and massively parallel SSU rRNA sequencing approaches to

253 characterize the skin microbiota of individuals with leprosy and attempted to determine

254 how it differs from the bacterial skin composition of healthy individuals. The

255 sequencing depth in this study revealed relatively rare members of the skin bacterial

256 community that collectively could have a negative implication on health.

257   Leprous skin lesion revealed four dominant phyla represented by,

258 Proteobacteria, Bacteroidetes Firmicutes and Actinobacteria. The same phyla were

259 found in skin from psoriasis and atopic dermatitis patients and from healthy persons

260 (*Gao et al., 2008; Costello et al., 2009; Grice et al., 2009; Kong et al., 2012; Blaser et*

261 *al., 2013*). However, the distribution of these phyla in the leprous lesion studied here

262 was distinct from that reported in these studies. Indeed, while Actinobacteria is the most

263 abundant and diverse phylum in healthy skin, with distribution ranging from 27% to

264 52% (*Costello et al., 2009; Grice et al., 2009; Blaser et al., 2013*) , in leprotic skin it

265 was markedly underrepresented (Fig. 4). Actinobacteria was also underrepresented

266 (37.3%) in psoriatic skin patches compared to healthy skin from the same patients

267 (47.8%) and from unaffected controls 47.6%; (*Gao et al., 2008*). As already suggested

268 by *Gao et al.* (*2008*) for psoriasis, the observed reduction in Actinobacteria

269 representation in the leprous lesion may be the result of disordered ecological niches of

270 the diseased skin, turning it inhospitable to these bacteria. Interestingly, in the leprous

271 lesion *Propionibacterium* and *Corynebacterium* were scarcely detected, in contrast to

272 their known dominant presence in normal skin (*Grice et al., 2009; Costello et al., 2009*)

273 Therefore, it is likely that Actinobacteria and in particular the *Propionibacterium* and

274 *Corynebacterium* genera may have a protective role in normal skin that is diminished in

275 leprous lesions. Lower representation of *Propionibacterium* species has also been

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276   observed in the psoriatic lesions (*Gao et al., 2008*). Moreover, it should be noted that

277   the absence of *M.leprae*-related sequences suggests that this taxon is not prevalent in

278   leprous lesions. Because the leprous lesions studied were histopathologically diagnosed,

279   the absence of *M. leprae*-related sequences deserves further attention. Although the

280   Firmicutes phylum was less abundant, *Streptococcus* was enriched in leprous lesion.

281   According to *Dekio et al.* (*2007*), they are considered to reside only in infected lesions

282   human skin. Another interesting finding was the low abundance of *Staphylococcus*

283   species, which densely colonize the skin, and has been considered a commensal in

284   healthy skin (*Iwase et al., 2010*).

285   Proteobacteria and Bacteroidetes, the two other major phyla inhabiting skin of

286   healthy persons, were significantly overrepresented in the leprous lesion (Fig. 4).

287   Indeed, in healthy persons the distribution of Proteobacteria ranges from 10 to 33% and

288   that of Bacteroidetes ranges from 2.4 to 10% (*Grice et al., 2009; Costello et al., 2009;*

289   *Gao et al., 2008; Blaser et al., 2013*).

290   Our data revealed that the *Burkholderia* (Sanger sequencing) and *Pseudomonas*

291   (V3-V4 tag) genera, were enriched and the most abundant. We also found the minor

292   genera *Nocardioides*, *Lysinibacillus*, *Geobacillus*, *Rhodoplanes*, *Gallionella*,

293   *Phycicoccus*, and *Dyadobacter*; to our knowledge the first identification of such

294   members in human skin. It is possible that leprous lesions impair the skin barrier

295   protection and facilitate the access of bacteria normally absent in healthy skin.

296   Here we describe for the first time the taxonomic diversity of the microbiota of

297   the leprous lesion. Sanger and massively parallel sequencing of leprous lesions provided

298   the same phylum-level representation of human skin, that account for 99% of the 16S

299   rRNA gene sequences (Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes).

300   However, rare and different taxa arise due to a massive increase in the sequencing

depth. Our results extend the findings of others by demonstrating that leprous lesion harbors a phylum-level diversity much more thus far known from the healthy skin microbiota. Thus, our data indicate that the leprous lesion harbors a different microbiota profile compared to that of healthy skin. Significant shifts of the microbiota seem to favor the colonization of potentially pathogenic bacteria, negatively impacting the abundance of bacteria that populate healthy skin. The comprehensive current knowledge on complexity in the composition of the microbiota is raising speculation on its correlation with the evolution of this disease. Thus, instead of a single organism being the sole causative agent of a given pathology, as proposed by Koch, disease may be a result of complex interactions among the bacterial community and between the microbiota and its local environment. With this speculation in mind, the current study can be used as a baseline for further research aiming to determine the contribution of bacteria other than *M. leprae* in triggering leprosy. In any case, knowledge on the composition of the leprous lesion community may be relevant to future studies concerning the development of new treatment strategies.

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394

395 **FIGURE LEGENDS**

396

397 Figure 1: Rarefaction curves on the dataset of the samples from leprous skin lesion. A.

398 Sanger sequencing and B massively parallel sequencing.

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399   Figure 2: Relative abundance of taxa observed in bacterial 16S rRNA gene library from

400   leprous skin lesion, based on Sanger sequencing.

401

402   Figure 3: Phylogenetic tree, constructed using the neighbor-joining method, show the

403   affiliation of bacterial OTUs from leprous skin lesions.

404

405   Figure 4: Relative abundance of taxa observed in two leprous lesions samples, based on

406   massively parallel sequencing. V3-V4 tags are grouped into phylum. Each phylum bar

407   is broken down when a particular taxonomic group dominated the phylum. Other phyla

408   are: AC1, Armatimonadetes, Chlorobi, Cyanobacteria, Elusimicrobia, Fusobacteria,

409   Gemmatimonadetes, GN02, GN04, OD1, OP1, OP11, OP3, OP8, Planctomycetes,

410   Spirochaetes, TM7 and WS3.

411

Legend:
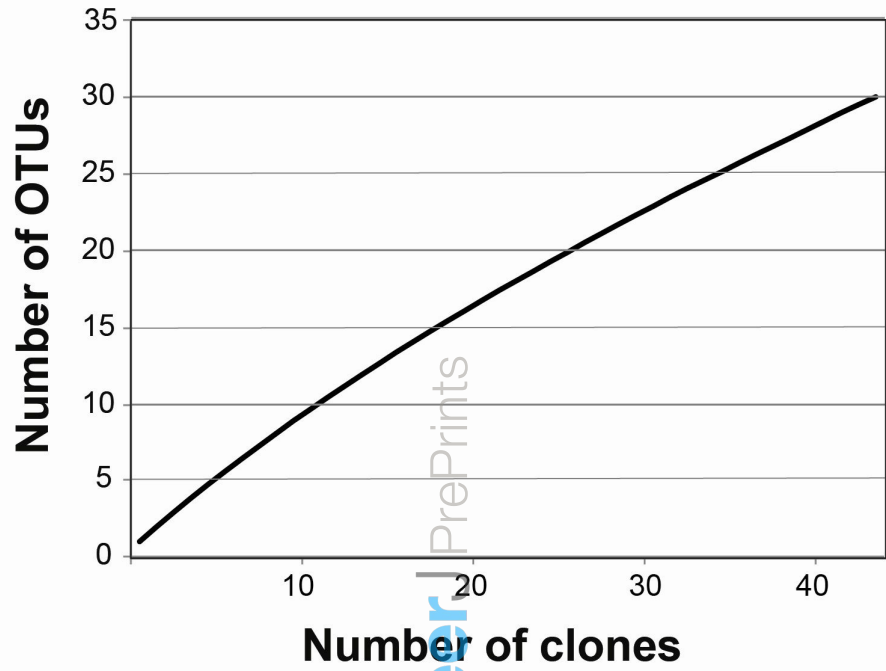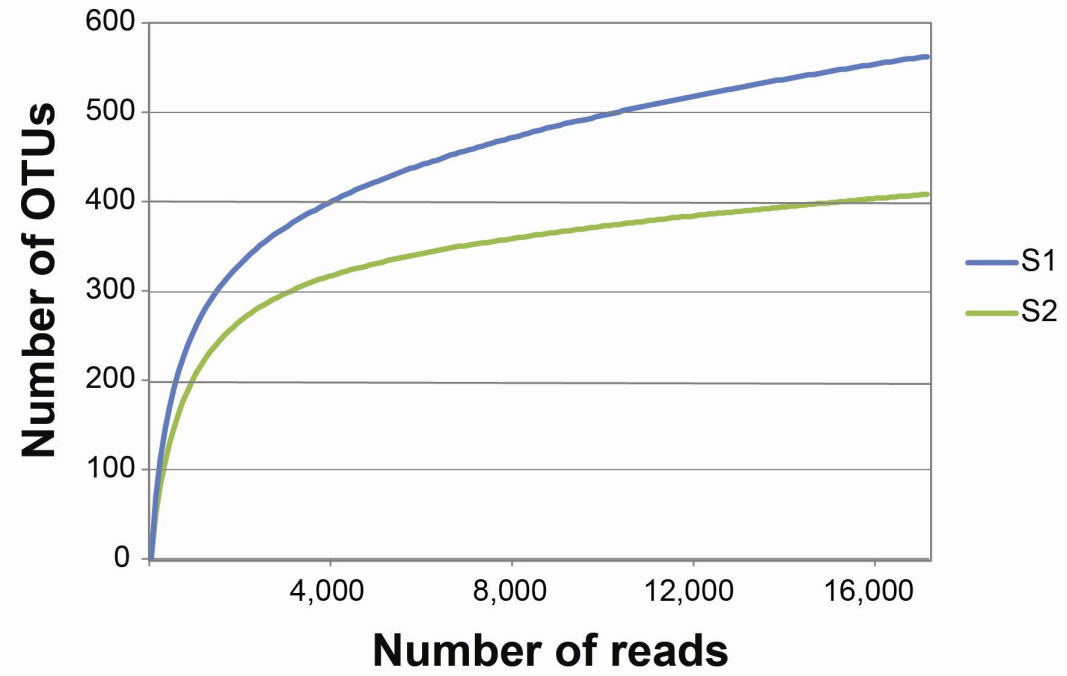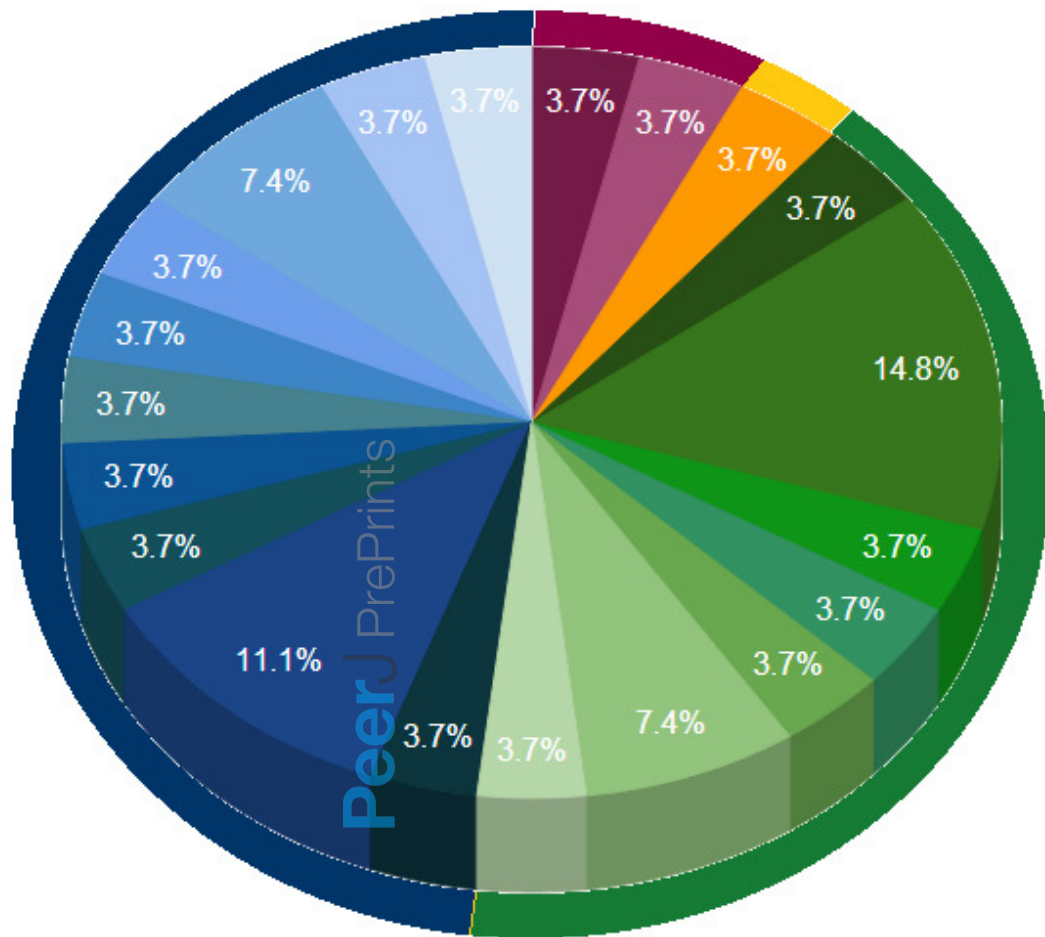
★ Actinobacteria ★ Bacteroidetes ★ Firmicutes ★ Proteobacteria

Propionibacterium | Dyadobacter | Staphylococcus | Klebsiella
Nocardioides | | Bacillus | Burkholderia
| | Lysinibacillus | Hydrogenophilus
| | Geobacillus | Pseudomonas
| | Bacillales | Achromobacter
| | Bacillaceae | Sphingomonas
| | Planococcaceae | Rhodoplanes
| | | Xanthomonadaceae
| | | Methylocystaceae
| | | Gammaproteobacteria