Multi-locus and long amplicon sequencing approach to study microbial diversity at species level using the MinION™ portable nanopore sequencer

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Abstract

Background: The miniaturised and portable DNA sequencer MinION™ has demonstrated great potential in different analyses such as genome-wide sequencing, pathogen outbreak detection and surveillance, human genome variability, and microbial diversity. In this study, we tested the ability of the MinION™ platform to perform long amplicon sequencing in order to design new approaches to study microbial diversity using a multi-locus approach.

Results: After compiling a robust database by parsing and extracting the *rrn* bacterial region from more than 67,000 complete or draft bacterial genomes, we demonstrated that the data obtained during sequencing of the long amplicon in the MinION™ device using R9 and R9.4 chemistries was sufficient to study two mock microbial communities in a multiplex manner and to almost completely reconstruct the microbial diversity contained in the HM782D and D6305 mock communities.

Conclusions: Although nanopore-based sequencing produces reads with lower per-base accuracy compared with other platforms, we presented a novel approach consisting of multi-locus and long amplicon sequencing using the MinION™ MkIb DNA sequencer and R9 and R9.4 chemistries that help to overcome the main disadvantage of this portable sequencing platform. Furthermore, the nanopore sequencing library constructed with the last releases of pore chemistry (R9.4) and sequencing kit (SQK-LSK108) permitted to retrieve the higher level of 1D read accuracy sufficient to characterize the microbial species present in each mock community analysed. Improvements in nanopore chemistry, such as minimising base-calling errors and new library protocols able to produce rapid 1D libraries, will provide more reliable information in near future. Such data will be useful for more

41 comprehensive and faster specific detection of microbial species and strains in complex
42 ecosystems.
43
44 **Keywords:** MinION; Nanopore sequencer; Ribosomal operon; Long amplicon sequencing;
45 Microbial diversity; Long-read sequencing

Background

During the last two years, DNA sequencing based on single-molecule technology has completely changed the perception of genomics for scientists working in a wide range of scientific fields. This new perspective is not only supported by the technology itself but also by the affordability of these sequencing instruments. In fact, unprecedentedly, Oxford Nanopore Technologies (ONT) released the first miniaturised and portable DNA sequencer in early 2014, within the framework of the MinION™ Access Programme. Recently, the MARC consortium (MinION Analysis and Reference Consortium) has published results related to the study of the reproducibility and global performance of the MinION™ platform. These results indicate that this platform is susceptible of a large stochastic variation, essentially derived from the wet-lab and MinION™ operative methods, but also that variability has minimal impact on data quality [1].

The coordinated and collaborative work and mutual feedback between industry and the scientific community have enabled ONT to develop rapidly towards improving its portable platform for DNA sequencing, minimizing the stochastic variation during DNA library preparation. Consequently, in late Autumn 2015, ONT released MkIb, the latest version of MinION™, and in April 2016 the fast mode chemistry (R9) was released, increasing the rate of sensing DNA strands from 30-70 to 280-500 bp/sec and reaching up to 95% of per-base accuracy in 2D reads (Clive G. Brown, CTO ONT, personal communication).

One of the most attractive capabilities of the MinION™ platform is the sequencing and assembly of complete bacterial genomes using exclusively nanopore reads [2] or through

hybrid approaches [3, 4]. Notwithstanding, the MinION™ platform has also been demonstrated useful in other relevant areas including: human genetic variant discovery [5, 6], detection of human pathogens [7, 8], detection of antibiotic resistance [9, 10], and microbial diversity [11, 12]. Regarding the latter, microbial diversity and taxonomic approaches are common and in high demand to analyse the microbiota associated to a wide variety of environment- and human-derived samples. However, these analyses are greatly limited by the short-read strategies commonly employed . Thanks to improvements in the chemistry of the most common, popular sequencing platforms in recent years, it is now possible to characterise microbial communities in detail, down to the family or even genus level, using genetic information derived from roughly 30% (~500nt) of the full 16S rRNA gene. Despite the massive coverage achieved with short-read methods, the limitation in terms of read length means taxonomic assignment at the species level is still unfeasible. For instance, taxonomy strategies based on short-reads from Illumina MiSeq platform offer a limited information that underestimates the microbial diversity of complex samples when compared with alternative approaches based on long DNA reads [13]. Consequently, implementation of long-read sequencing approaches to study larger fragments of marker genes will permit the design of new studies to provide evidence for the central role of precise bacterial species/strains in a great variety of microbial consortia. Recent studies at this regard have showed important advances in taxonomy analysis using long reads generated by single molecule technologies [11, 14, 15], indicating that the expansion or inclusion of more hypervariable regions in the analysis overcomes the disadvantage of working with error-prone DNA reads. With respect to the above, we have recently explored the performance of the MinION™ device. Our study demonstrates that data obtained from sequencing nearly full-length 16S rRNA gene amplicons is feasible to study microbial

93 communities through nanopore technology [11]. We wanted to move a step forward in this
94 type of strategy, thus gaining more specificity when including several hypervariable
95 markers in the analysis, at sequence and structural level, by designing a multi-locus and
96 long amplicon sequencing method to study microbial diversity. At the same time, we also
97 wanted to explore the affordability of the MinION™ technology to perform microbial
98 diversity analyses by multiplexing several samples in one single MinION™ flowcell.
99 Accordingly, here we present a study of the 16S, 23S, and the internal transcribed spacer
100 (ITS, that frequently encodes tRNA genes) simultaneous sequencing, using the MinION™
101 MkIb device and R9 chemistry, with prior generation of ~4.5kb DNA fragments by
102 amplifying the nearly full-length operon encoding the two larger ribosomal RNA genes in
103 bacteria, the *rrn* region (*rrn* hereinafter). We have studied the *rrn* of two mock microbial
104 communities, composed of genomic DNA from 20 and 8 different bacterial species,
105 obtained respectively from BEI Resources and ZYMO Research Corp., using the
106 MinION™ sequencing platform in multiplex configuration.
107

108 ## Data description

109 The R9 raw data collected in this experiment was obtained as fast5 files using
110 MinKNOW™ v0.51.3.40 (Oxford Nanopore Technologies) after conversion of electric
111 signals into base calls via the Metrichor™ agent v2.40.17 and the Barcoding plus 2D
112 Basecalling RNN for SQK-NSK007 workflow v1.107; whereas the R9.4 raw data was
113 generated by MinKNOW™ v1.5.5 (Oxford Nanopore Technologies) with the respective
114 local basecalling algorithm implemented in that version of the MinION™ controller
115 software. Base-called data passing quality control and filtering were downloaded and data

was converted to fasta format using the *poRe* package [16]. Fast5 raw data can be accessed at the European Nucleotide Archive (ENA) under the project ID PRJEB15264. Only two data sets were generated after a sequencing run of MinION™ MkIb.

Analysis

Defining the arrangement of the rrn region. The complete or partial gene sequence of the RNA attached to the small subunit of the ribosome is classically used to perform taxonomy and diversity analysis in complex samples containing hundreds of microbial species. In the case of bacterial species, the 16S rRNA gene is the most widely used DNA marker for taxonomic identification of a particular species, given the relatively high number of hypervariable regions (V1 to V9) present across its sequence. Nowadays, it is possible to study the complete or almost full-length sequence of the 16S rRNA molecule thanks to single-molecule sequencing approaches [11, 14, 15, 17]. The identification of complex microbial communities at species-level with raw data obtained from MinION™ or PacBIO platforms is improving; however, uncertainty in taxonomic assignation is still noteworthy given the high proportion of errors in their reads. While future technical advances may improve the quality of DNA reads generated by third generation sequencing devices, new strategies can also be adopted to enhance the performance of these approaches. Consequently, we postulate that a good example of this is to study a common multi-locus region of the bacterial genome, which enables the simultaneous study of more variable regions and locus arrangements (sequence and structural variation), such as the operon encoding the ribosomal RNA. Using a complex sample where hundreds of microbial species are potentially present (DNA from human faeces) we carried out preliminary

experiments to amplify the *rrn*. We observed that from the hypothetical configurations envisaged for the *rrn* (Figure 1A), we only obtained a clear amplification using the primer pairs S-D-Bact-0008-c-S-20 and 23S-2241R, indicating that the *rrn* preferentially seems to be transcriptionally arranged as follows: 16S-ITS-23S. A detailed evaluation of the fragment size determined that main PCR products ranged from 4.3 to 5.4kbp (Figure 1B-D), being consistent with the expected size of PCR products amplifying the 16S, ITS, and 23S regions from several microbial species. The next step involved designing a multiplex sequencing approach to try to analyse more than one sample per sequencing run in one flowcell of MinION^{TM}; therefore, the primers were re-designed to include a distinctive barcode region at 5´ (Table 1). During PCR of the *rrn* we tagged the amplicon derived from the mock community HM782D with the barcode *bc01* in a dual manner, whereas the amplicons derived from sample D6305 were tagged with barcode *bc08* in similar way. Parallel experiments were conducted on HM782D and D6305 DNA, with comparative aims, using a conventional protocol of microbial diversity analysis and consisting of the V4-V5 16S amplicon sequencing by Illumina MiSeq paired-end approach (see methods).

The reference database. One of the major handicaps when proposing this new *rrn* region to be used for taxonomy analysis is the need to compile a reference database to compare the reads produced by MinION™ device. Therefore, we proceeded to parse the genetic information of over 67,000 bacterial genomes whose sequences are publicly available in GenBank at NCBI. In this way, we retrieve and compile more than 47,000 *rrn* sequences that were subject of a clustering analysis to reduce the level of redundancy and to disclose the variability intrinsically associated to the *rrn* itself and to its individual components as well (Figure 2).

After normalization of cluster numbers against the median size of respective regions analyzed and referenced against the numbers obtained for 16S region at 97% sequence identity, we found that *rrn* region comprising the 16S, ITS, and 23S coding regions exhibits more than 4-fold more variation than that observed for the 16S molecule alone (at 100% sequence identity). As expected, the 23S region exhibited more diversity by containing more hypervariable regions than 16S region and getting almost 2-fold more diversity. Strikingly, the ITS regions showed similar levels of genetic diversity despite to have almost one fourth of the size of 16S region in average. When parsing the genetic information of over 67,000 bacterial genomes, we observed the ITS region frequently encodes one or several tRNA genes and it possess a high variability in terms of length as well. Consequently, the variability observed in the *rrn* was the largest observed and thought to be meaningful for the aims of this study. We obtaining data supporting the above notion by searching the number of *rrn* clusters (at 100% identity) matching with the most predominant species in the database, thus retrieving 1,713, 1,276, and 1,273 *rrn* clusters annotated for *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, respectively. In consequence, the *rrn* is able to accumulate enough sequence variability to discern taxonomy even at strain level.

Performance of the R9 chemistry. Once we could compile a reference database for comparison aims, we proceeded with the amplicon library construction and sequencing run obtaining raw data consisting of 17,038 reads and almost all were classified as 1D reads. For general knowledge, the DNA reads derived from the MinION™ device can be classified into three types: '1D template', '1D complement', and '2D' reads. The latter, 2D reads, are products of aligning and merging sequences from the template (read from leader

187 adapter) and complement reads (a second adapter called hairpin or HP adapter must be
188 generated), produced from the same DNA fragment. These contain a lower error rate,
189 owing to strand comparison and mismatch correction. In addition to the technical issues
190 indicative of a bad ligation of the HP adapter, we obtained 93% of reads (~15,900 reads)
191 during the first 16h of run; thus, we obtained lower sequencing performance after re-
192 loading with the second aliquot of the sequencing library and extended the run for another
193 24h (40h in sum). The fasta sequences were filtered by retaining those between 1,500 and
194 7,000 nt in length, obtaining at least enough sequence information to compare a DNA
195 sequence equivalent to the 16S rRNA gene length. After this filtering step, we retained 72%
196 of sequences (12,278) and then we performed the respective barcode splitting. For this
197 purpose, we modified the default parameters of the "split_barcodes.pl" perl script (Oxford
198 Nanopore Technologies) by incorporating the information of the extended barcodes (Table
199 1), rather than the barcode information alone, and simultaneously increased the stringency
200 parameter to 25 (14 by default). Afterwards the concatenation of reads were obtained from
201 respective forward and reverse extended barcodes, then we retrieved a total of 2,019 (52%
202 from forward and 48% from reverse barcodes) and 1,519 (53% from forward and 47% from
203 reverse barcodes) 1D reads for HM782D and D6305 mock communities, respectively.
204 Read-mapping was performed against the *rrn* database, compiling more than 22,000 *rrn*
205 regions, retrieved from more than 67,000 genomes available in GenBank (see Availability
206 of supporting data). The taxonomy associated to the best hit based on the competitive
207 alignment score followed by filtering steps (see methods) was used to determine the
208 structure of each mock community. The MinION™ sequencing data produced the microbial
209 structure presented in Figure 3 for the mock communities HM782D and D6305,
210 respectively.

211

212 Figure 3 shows the bacterial species and their respective relative proportions retrieved from
213 the analysis of the mock communities HM782D and D6305, respectively. With respect to
214 the HM782D mock community, we were able to recover 20 representative species,
215 accounting for 16 out of 20 species present in that artificial community (Figure 3A).
216 However, the remaining four species that apparently are absent in this community have a
217 close relationship to others detected correctly, namely *Bacillus subtilis, Bacillus*
218 *thuringensis, Bacillus anthracis,* and *Propionibacterium sp.* Furthermore, we were unable
219 to report the presence of just four species present in HM782D because proportions of
220 *Rhodobacter sphaeroides* and *Actinomyces odontolyticus* were below the predominance
221 threshold (1%), being present in 0.25 and 0.12%, respectively. Similarly, other 40 different
222 species but close to that present in the HM782D mock community (*Bacillus* spp.,
223 *Streptococcus* spp. *Clostriudium* spp., *Neisseria* spp., *Staphylococcus* spp, and *Listeria*
224 spp.) had minor representation in data derived from *rrn* sequencing. With respect to
225 *Rhodobacter sphaeroides* and *Actinomyces odontolyticus* lower proportions, we have
226 previously demonstrated that the low levels of 16S reads are a consequence of
227 amplification bias derived from the PCR reaction and not from sequencing itself [11]. In
228 this case, the new primer pair used to generate the long amplicons would seem to work
229 more efficiently than those previously used, but apparently they still present issues at
230 bacterial coverage level. When we revised the whole taxonomy contained in our *rrn*
231 database, the compiling of non *rrn* regions for *Deinococcus radiodurans* and *Helicobacter*
232 *pylori* partially explained the lack of these species in HM782D analysed by the present
233 approach. However, a new alignment process using individual 16S and 23S rRNA
234 sequences obtained from GenBank and including those for *D. radiodurans* and *H. pylori*,

235 respectively, demonstrated that at least *D. radiodurans* could be identified in a higher
236 proportion than *A. odontolyticus* and *R. sphaeroides*, albeit in a lower proportion than our
237 predominance threshold. Regarding the results obtained from the D6305 mock community,
238 we found a total of 10 bacterial species present in this mixed DNA sample, eight of them
239 matched the expected structure of the community, and additionally 18 close species had
240 minor representation (*Bacillus* spp., *Enterococcus* spp., *Klebsiella* spp., *Lactobacillus* spp.,
241 *Streptococcus* spp., and *Staphylococcus* spp.). Using the MinION™ data we were able to
242 recover 100% of the species present in this sample and the two additional members
243 identified also have a close relationship within the *Bacillus* genus, as observed in the
244 HM782D sample (Figure 3B). We have determined that coverage needed to retrieve all
245 expected species in a non-even mock community with an abundance above 1% is ~13X in
246 terms of the number of species of that community.
247
248 When compared to reference values and proportions theoretically expected for the species
249 present in the two mock communities, we observed some deviations that were greater in
250 certain species. Particularly, in the HM782D sample the lowest coverage biases were
251 observed for *Actinomyces odontolyticus* (-5.36), *Rhodobacter sphaeroides* (-4.36), and
252 *Enterococcus faecalis* (-2.04). This indicates that such species, in addition to *D.*
253 *radiodurans* and *H. pylori*, are more difficult to detect with the primers and PCR used here.
254 By contrast, *Escherichia coli* (1.79) seems to be preferentially amplified, given that this
255 species exhibited the highest positive coverage bias value (Figure 3C). We again found that
256 coverage bias is linearly correlated with PCR products generated by quantifying *E.coli, L.*
257 *gasseri*, and *B. vulgatus* amplicons (Pearson's r = 0.82, p = 0.047), data indicating that there
258 are not major issues during taxonomy assignation by over-representation of certain species

in the reference database. The values obtained for D6305 were more homogeneous, and the lowest coverage bias was observed for *Lactobacillus fermentum* (-2.18) (Figure 3D). Additional analysis indicated that there was not significant correlation between coverage bias and GC content in *rrn*. Although the low coverage bias for some species can be solved by selecting another pair of primers, the ability to recover almost all of them, at least in a low proportion, in itself represents an important attribute of this approach for inter-sample comparisons. Interestingly, we observed a similar pattern of overrepresentation of *Bacillus* spp. sequences (>50%) in D6305 sample but not for *Escherichia* spp. sequences (~4%) in the HM782D mock community when Illumina MiSeq data was assessed (Figure 3C-D).

The high error rate of the 1D reads (ranging between 70 and 87% sequence identity, according to high quality alignments) makes barcoding de-multiplexing a difficult task in nanopore data. However, our results indicate that with the configuration and parameters presented here we could efficiently distinguish the reads generated from HM782D and D6305 amplicons. As a consequence, the performance of this long amplicon approach to properly assign microbial communities to samples was efficiently assisted by the parameters during the de-multiplexing process that were central to discern reads obtained from respective samples multiplexed in the MinION flowcell. For instance, the distribution of reads matching with close related species such as *Lactobacillus gasseri* and *Lactobacillus fermentum*, contained distinctively in HM782D and D6305 samples, was indicative of the adequate execution of the de-multiplexing pipeline. The above was also exemplified for *Salmonella enterica* sequences that were determined only in D6305 despite its close relationship with *E. coli* at the 16S and 23S sequence level (close to 100%). Regarding the latter, the multiple sequence alignment built with *rrn* regions from both

283 species was inspected directly distinguishing the ITS as the major source of variation
284 between the two species. Indeed, this was corroborated by the comparative analysis
285 performed during the clustering step of the reference samples to create our *rrn* database.
286
287 *Performance of R9.4 chemistry*. During the course of the present work the MinION R9.4
288 chemistry was delivered in Autumn 2016. Therefore, we wanted to perform a replicate
289 experiment using this type of chemistry in order to disclose how much improvement our
290 approach would gain in terms of sensibility and specificity. With only 3h run we observed a
291 notable improvement of throughput and per-base accuracy and the MinION™ produced
292 almost 40,000 reads with a predominant QScore distribution between 8 and 12 suggesting a
293 theoretical error rate of reads between 0.15 to 0.06, respectively, lower than obtained from
294 R9 reads (0.25 to 0.15). After compiling all sequences in a fasta file, we proceeded to
295 perform filtering in equal manner than previously done for R9 data. Consequently, we
296 retained more than 33,000 reads (86%) for further processing and taxonomy assignment.
297 The major results from comparison among R9 and R9.4 runs are summarized in the Table
298 2. As expected, the R9.4 dataset was more accurate and its reads showed a lower per-base
299 error rate, therefore, the taxonomy analysis based on this reads would be more precise than
300 observed with R9 reads. Globally, the results obtained from R9.4 chemistry are very similar
301 than those observed with R9 chemistry but the level of uncertainty was diminished by
302 reducing the number of close species to that contained in respective mock communities
303 exhibiting very low abundance (<1%), thus decreasing from 40 species to 15 for the
304 HM782D and from 18 to 16 for the D6305. We were unable again to recover *D.*
305 *radiodurans* and *H. pylori* reads but we improved the sensitivity for *A. odontolyticus* and *R.*
306 *sphaeroides* (Figure 3C and 3D), whose relative proportions were almost duplicated in

307 R9.4 data (*R. sphaeroides* = 0.44%, *A. odontolyticus* = 0.31%). We compared the
308 respective proportions obtained from R9 and R9.4 chemistries obtaining consistent results
309 (Figure 3E) indicating that our approach is reproducible with no major changes despite the
310 different chemistry and kits for library preparation using during both sequencing runs.
311
312 *Comparison with Illumina MiSeq data*. The Illumina MiSeq data obtained after sequencing
313 the V4-V5 16S region permitted to characterize the genus distribution in the HM782D
314 sample with the RDP Classifier. As a result, we compiled distribution of all 17 genus
315 represented in the HM782D mock community (Supplementary Material 1) and 4 additional
316 genus with very low abundance (2 reads / 8,409 assigned). Moreover, when a OTU-picking
317 approach was conducted we recovered 41 OTUs whose identity was evaluated in the SINA
318 server (Supplementary Material 2). Globally, we recovered taxonomy identification of all
319 genus expected but only three species were well identified based on the Greengenes
320 taxonomy (*H. pylori, P. acnes,* and *R. sphaeroides*) whereas one was wrongly identified
321 (*Neisseria cinerea*). For the D6305 mock community we could recover the eight different
322 component at genus level of this mock community (Supplementary Material 1) plus seven
323 additional and not related genus with very low abundance (< 7 reads / 8,046 assigned). At
324 OTUs level, we retrieved a total of 14 sequences whose taxonomy identification is
325 presented in the Supplementary Material 3. In this case, only *S. enterica* could be identified
326 at species level. Given that data derived from this short read approach normally cannot
327 reach a reliable taxonomy assignment down to species level, we proceed to make
328 comparisons with R9 and R9.4 data by compiling these last information to genus level in
329 order to evaluate the performance of our approach with a commonly used procedure. In the
330 Supplementary Material 1 and Table 3 a comparison in terms of the relative read proportion

and coverage bias is depicted. We observed no larger deviations in data retrieved with MinION regarding those numbers obtained with conventional approaches such as study of V4-V5 regions with MiSeq platform. Interestingly, we observed similar pattern of important negative coverage bias in all three approaches for *Actinomyces* spp., *Enterococcus* spp., and *Rhodobacter* spp. species in the HM782D community and for *Lactobacillus* spp., and *Listeria* spp., in the D6305 community, then suggesting that species of such genera are equally underrepresented no matter the type of amplicon, sequencing platform, or sequencing chemistry of study. Conversely, only the *Bacillus* spp. species from the D6305 exhibited large positive coverage bias values in all three approaches. Globally, all methods compared to study microbial communities at this level have a pattern of underrepresentation for all species present in the mock communities given the average and median values obtained. Moreover, the MiSeq V4-V5 approach also showed important coverage bias indicating this issue is not strictly associated with the MinION™ based approach presented in here and probably it is inherent to the amplification process of target DNA. Finally, correlation tests indicate that despite of coverage bias observed all configurations used to study the mock communities replicate fairly well the composition of the mock communities and that data obtained from R9 and R9.4 experiments show a slight improvement at this regard with no major differences when compared with data from MiSeq platform (Table 3).

Discussion

The inventory of microbial species based on 16S rDNA sequencing is frequently used in biomedical research to determine microbial organisms inhabiting the human body and their

354 relationship with disease. Recently, third-generation of DNA sequencing platforms have
355 developed rapidly, facilitating the identification of microbial species and overcoming the
356 read-length issues inherent to second-generation sequencing methods. These advances
357 allow researchers to infer taxonomy and analyse diversity from the almost full-length
358 bacterial 16S rRNA sequence [11, 14, 15, 17]. Particularly, the ONT platform deserves
359 special attention given its portability and its fast development since the MinION™ became
360 available in 2014. Notwithstanding, this technology is susceptible to a large stochastic
361 variation, essentially derived from the wet-lab methods [1]. We corroborated this issue by
362 obtaining a sequencing run where the raw data predominantly consisted of 1D reads as a
363 consequence of the HP adapter ligation failure, despite following the manufacturer's
364 instructions. However, we were able to develop an efficient analysis protocol where the
365 higher read quality offered by R9 chemistry and the updated Metrichor basecaller protocol
366 proved pivotal to obtain 1D reads with a range of identity between 70 and 86%, with
367 sufficient per-base accuracy to successfully perform the taxonomic analyses described
368 herein. Moreover, during the course of this study the R9.4 flowcells were released and we
369 were able to replicate our approach using this improved pore chemistry and the SQK-
370 LSK108 for 1D libraries obtaining reads with sequence identity up to 92%.
371
372 Our preliminary results indicated that the *rrn* region in bacteria preferentially has a unique
373 conformation (with the transcriptional arrangement of 16S-ITS-23S) and we could amplify
374 this ~4.5Kbp region with the selected S-D-Bact-0008-c-S-20 and 23S-2241R primer pair.
375 Once we were able to distinguish the feasibility to amplify the *rrn*, our approach comprised
376 the study of two different mock communities in a multiplex manner, to be combined in one
377 single MinION™ flowcell. By designing the respective forward and reverse primers tagged

with specific barcodes recommended by ONT, we were able to retrieve extended barcode-associated reads, in spite of the large proportion of per-base errors contained in these types of reads. Using MinION™ data based on multi-locus markers and long amplicon sequencing, we could reconstruct the structure of two commercially available mock communities. Although the expected proportions of some species in each community exhibited an important coverage bias, we were able to recover 80% (HM782D) and 100% (D6305) of bacterial species from the respective mock communities. Consequently, future analyses should be conducted to find an appropriate PCR approach using primers with a higher coverage for bacterial species.

We have analysed a great amount of genetic information with the aim of compiling a valuable database containing the genetic information for the *rrn* present in over 67,000 draft and complete bacterial genomes. The global length distributions in the region indicated that the *rrn* was 4,993 ± 187 bp in length whereas the 16S, ITS, and 23S sub-regions were 1,612 ± 75, 488 ± 186, and 3,036 ± 160 bp in length, respectively. Using this genetic information of the *rrn* and clustered at 100% of sequence identity enabled us to establish a multi-locus marker able to discriminate the taxonomy of two mock communities containing very close species. The latter was possible given that simultaneous analysis of the 16S, ITS, and 23S molecules offered almost 40-fold more diversity that studying the 16S, ITS, or 23S sequences separately and at 97% sequence identity. Moreover, the ITS was distinguished individually as an important variable genetic region in terms of sequence and length. Furthermore, it contributes notably to the higher variability observed in the *rrn* region, a fact evidenced in previous studies [18-21]. The accumulation of a larger number of variable sites in the *rrn* region, together with the particular structural variation of the ITS

to potentially accommodate and encode tRNA genes, are thought to be central to discriminating bacterial species, despite the large proportion of per-base errors contained in MinION™ reads. Our data indicate that our MinION reads produce alignments with averaged length of 2,463 and 3,191 bases for HM782D and D6305, respectively, using R9 chemistry and 4,173 and 4,115 bases for HM782D and D6305, respectively, using R9.4 chemistry. Consequently, the taxonomy assignment was predominantly based on the variability of more than two out of the three markers included in the *rrn*, no matter if reads were produced from the 16S or 23S edges of *rrn* amplicons. We expect this type of analysis will likely become more accurate over time as nanopore chemistry improves in near future, with the concomitant increase in throughput, which is pivotal to disclose the hundreds of species present in complex microbial communities for analysis in human or environmental studies. Therefore, the multi-locus, long and multiplex methods described here represent a promising analysis routine for microbial and pathogen identification, relying on the sequence variation accumulated in approximately 5kbp of DNA, roughly accounting for the assessment of 1.25% of an average bacterial genome (~4Mbp). Notwithstanding, we cannot obviate that the current state of this approach presents some limitations in terms of the completeness of the *rrn* database created as well as the efficiency of the primers used to generate the long amplicons that have to be revisited in order to improve and increase the coverage of bacterial species. At date, our database include *rrn* sequences from 2,479 different species grouped into 918 different genus. In consequence, urgent studies must be undertaken to generate a more complete database including the *rrn* genomic information from species inhibiting complex and real samples such as those derived from human body.

Methods

Bacterial DNA and rrn amplicons

The complex DNA sample for preliminary studies of *rrn* region arrangement consisted of DNA isolated from faeces, kindly donated by a healthy volunteer upon informed consent. An aliquot of 200 mg of human faeces was used to isolate microbial DNA using the QIAamp DNA Stool Mini Kit (Qiagen) and following the manufacturer's instructions. Finally, DNA was eluted in 100 µL nuclease-free water and a DNA aliquot at 20 ng/µL was prepared for PCR reaction using the primer pairs S-D-Bact-0008-c-S-20 and 23S-2241R or 23S-129F and S-D-Bact-1391-a-A-17 for testing configurations shown in Figure 1A (Table 1). The band size was analysed using the Java-based GelAnalyzer tool (www.gelanalyzer.com). Genomic DNA for the reference mock microbial communities was kindly donated by BEI Resources (http://www.beiresources.org) and ZYMO Research Corp (http://www.zymoresearch.com). The composition of the mock communities was as follows: i) HM782D is a genomic DNA mixture of 20 bacterial species containing equimolar ribosomal RNA operon counts (100,000 copies per organism per µL), as indicated by the manufacturer; and ii) ZymoBIOMICS Cat No. D6305 (D6305 hereinafter) is a genomic DNA mixture of eight bacterial species (and two fungal species) presented in equimolar amounts of DNA. According to manufacturers' instructions, 1 µL of DNA from each mock community was used to amplify all the genes contained in the *rrn*. DNA was amplified in triplicate by 27 PCR cycles at 95ºC for 30 s, 49ºC for 15 s, and 72ºC for 210 s. Phusion High-Fidelity Taq Polymerase (Thermo Scientific) and the primers S-D-Bact-0008-c-S-20 (mapping on 5' of 16S gene) and 23S-2241R (mapping on 3' of 23S gene), which target a wide range of bacterial 16S rRNA genes [22, 23]. For the Illumina MiSeq

448 sequencing the V4-V5 hypervariable regions from bacterial 16S rRNA gene were amplified
449 using 1 µL of DNA from each mock community and 25 PCR cycles at 95ºC for 20 s, 40ºC
450 for 30 s, and 72ºC for 20 s. Phusion High-Fidelity Taq Polymerase (Thermo Scientific) and
451 the 6-mer barcoded primers S-D-Bact-0563-a-S-15 (AYTGGGYDTAAAGNG) and S-D-
452 Bact-0907-a-A-20 (CCGTCAATTYMTTTRAGTTT). As we wished to multiplex the
453 sequencing of both mock communities into one single MinION™ flowcell, we designed a
454 dual-barcode approach where respective primers were synthesized and fused with two
455 different barcodes recommended by ONT (Table 1). Amplicons consisted of ~4.5kbp blunt-
456 end fragments for MinION approach and ~380bp for Illumina MiSeq approach, and those
457 were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE
458 Healthcare). Amplicon DNA was quantified using a Qubit 3.0 fluorometer (Life
459 Technologies). Quantification of certain PCR products to correlate with sequencing
460 coverage bias by qPCR was assessed according to previously described [11].
461
462 *Amplicon DNA library preparation*
463 The Genomic DNA Sequencing Kit SQK-MAP006 was ordered from ONT and used to
464 prepare the amplicon library for loading into the MinION™. Approximately 0.9 µg of
465 amplicon DNA (0.3 per mock community plus 0.3 µg of an extra query sample consisting
466 amplicons obtained from a genomic DNA mix of several uncharacterized microbial
467 isolates) were processed for end repair using the NEBNextUltra II End Repair/dA-tailing
468 Module (New England Biolabs), and followed by purification using Agencourt AMPure
469 XP beads (Beckman Coulter) and washing twice with 1 volume of fresh 70% ethanol.
470 Subsequently, and according to the manufacturer's suggestions, we used 0.2 pmol of the

purified amplicon DNA (~594 ng, assuming fragments of 4.5kbp in length) to perform the adapter ligation step. Ten µL of adapter mix, 2 µL of HP adapter, and 50 µl of Blunt/TA ligase master mix (New England Biolabs) were added in that order to the 38 µl end-repaired amplicon DNA. The reaction was incubated at room temperature for 15 minutes, 1 µL HP Tether was added and incubated for an additional 10 minutes at room temperature. The adapter-ligated amplicon was recovered using MyOne C1-beads (Life Technologies) and rinsed with washing buffer provided with the Genomic DNA Sequencing Kit SQK-MAP006 (Oxford Nanopore Technologies). Finally, the sample was eluted from the MyOne C1-beads by adding 25 µL of elution buffer and incubating for 10 minutes at 37°C before pelleting in a magnetic rack. The R9.4 sequencing library was obtained by processing of 600 ng of purified amplicon DNA (0.15 per mock community plus 0.15 µg of two extra query sample consisting amplicons obtained from a genomic DNA mix of several uncharacterized microbial isolates) with the SQK-LSK108 (Oxford Nanopore Technologies) sequencing for 1D reads and following the manufacturer's instructions. Briefly, the 600 ng of amplicon DNA diluted in 50 µL nuclease-free water were processed for end repair using the NEBNextUltra II End Repair/dA-tailing Module (New England Biolabs), and followed by purification using Agencourt AMPure XP beads (Beckman Coulter) and washing twice with 200 µL volume of fresh 70% ethanol. The ligation step was performed with 30 µL of DNA end-prepped, 20 µL adapter mix, and 50 µl of Blunt/TA ligase master mix (New England Biolabs). The reaction was incubated at room temperature for 15 minutes at room temperature. The adapter-ligated amplicon was recovered again Agencourt AMPure XP beads (Beckman Coulter), washing twice with the ABB buffer supplied into the SQK-LSK1008 sequencing kit (Oxford Nanopore Technologies), and

494 eluted from Agencourt AMPure XP beads by adding 25 µL of elution buffer and incubating
495 for 10 minutes at 37°C before pelleting in a magnetic rack. Samples for Illumina MiSeq
496 approach were sent to the National Center for Genomic Analaysis (CNAG, Spain) for
497 multiplex sequencing in one lane of MiSeq instrument with 2x300 paired-end
498 configuration.
499
500 *Flowcell set-up*
501 A brand new, sealed R9 flowcell was acquired from ONT and stored at 4°C before use. The
502 flowcell was fitted to the MinION™ MkIb prior to loading the sequencing mix, ensuring
503 good thermal contact. The R9 flowcell was primed twice using 71 µL premixed nuclease-
504 free water, 75 µL 2x running buffer, and 4 µL fuel mix. At least 10 minutes were required
505 to equilibrate the flowcell before each round of priming and before final DNA library
506 loading. For the replicate experiment, a R9.4 flowcell was fitted to the MinION™ MkIb
507 prior to loading the sequencing mix, ensuring good thermal contact. The R9.4 flowcell was
508 primed with 800 µL of running buffer (0.5 mL nuclerase-free water plus 0.5 mL RBF
509 buffer). At least 10 minutes were required to equilibrate the flowcell and then the remaining
510 200 µL of running buffer were injected into the R9.4 flowcell with the SpotON port
511 opened.
512
513 *Amplicon DNA sequencing*
514 The sequencing mix was prepared with 59 µl nuclease-free water, 75 µl 2x running buffer,
515 12 µL DNA library, and 4 µL fuel mix. A standard 48-hour sequencing protocol was
516 initiated using the MinKNOW™ v0.51.3.40. Base-calling was performed through data

517 transference using the Metrichor™ agent v2.40.17 and the Barcoding plus 2D Basecalling
518 RNN for SQK-NSK007 workflow v1.107. During the sequencing run, one additional
519 freshly diluted aliquot of DNA library was loaded after 16 hours of initial input. The raw
520 sequencing data derived from the two mock communities studied here was expected to
521 account two-thirds of the data produced by the R9 flowcell used. The R9.4 run was
522 performed with 75 µL DNA library (37.5 µL, RBF buffer, 25.5 µL LLB, 12 µL DNA
523 library) loaded into the R9.4 flowcell through the SpotON port. A standard 48-hour
524 sequencing protocol was initiated using the MinKNOW™ v1.5.5 with the respective local
525 basecalling algorithm implemented in the MinKNOW™ software. The R9.4 raw data was
526 generated during only 3h sequencing run.
527
528 *The rrn database*
529 We built a database containing the genetic information for the 16S and 23S rRNA genes
530 and the ITS sequence in all the complete and draft bacterial genomes available in the NCBI
531 database (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/bacteria). A total of 67,199 genomes
532 were analysed by downloading the "*fna*" files and parsing for rRNA genes into the
533 respective "*gff*" annotation file. Chromosome coordinates for *rrn* regions were parsed and
534 used to extract such a DNA sequences from complete chromosomes or DNA contigs
535 assembled. The resulting *rrn* sequences were analysed and the length distribution was
536 assessed. We retrieved a total of 47,698 *rrn* sequences with an average of 4,993 nt in
537 length. By selecting the size distribution equal to the 99th percentile (two-sided), we
538 discarded potential incomplete or aberrant annotated *rrn* sequences and observed that *rrn*
539 sequences can be found between 4,196 and 5,790nt; under these boundaries, our *rrn*

database finally accounted for a total of 46,920 sequences. Equivalent databases were built by parsing the respective *rrn* sequences with the RNammer tool to discriminate the 16S, ITS, and 23S rRNA sequences [24]. To remove the level of redundancy of our *rrn* database and to maintain the potential discriminatory power at strain level, we performed clustering analysis using USEARCH v8 tool for sequence analysis and the option *-otu_radius_pct* equal 0 [25], thus obtaining a total of 22,350 reference sequences. For comparative aims, the *rrn* database and the 16S, ITS, and 23S databases were also analysed using the option *-otu_radius_pct* with values ranging from 1 to 3. For accessing to *rrn* database and the respective species annotation, see Availability of supporting data.

MinION data analysis

Read-mapping was performed using the LAST aligner v.189 [26] with parameters -q1 -b1 -Q0 -a1 -r1. Each 1D read was compared in a competitive way against the entire *rrn* database and the best hit was selected by obtaining the highest alignment score. Alignment length as well as alignment coordinates in target and query sequences were parsed from the LAST output and the sequence identity between matched regions was calculated using the python *Levenshtein* distance package. An iterative processing was used to determine thresholds for detection by evaluating the taxonomy distribution with reads subsampling and different levels of sequence identity in top scored alignments. High quality alignments were selected by filtering out those with identity values up to the 50th percentile of the distribution of identity values of all reads per sample (~69%) in the R9 run. Therefore, taxonomy assignment was based exclusively on alignments with $\geq$ 70% identity. For data derived from R9.4 chemistry, high quality alignments were selected by filtering out those with identity values up to 25th percentile of the distribution, thus retaining alignments with

564 ≥ 81% identity. Basic stats, distributions, filtering, and comparisons were performed in R
565 v3.2.0 (https://cran.r-project.org). For relative quantification of species the singletons were
566 removed and the microbial species considered to be predominantly present in the mock
567 communities were those with a relative a proportion ≥ 1%, a value that demonstrated to be
568 discriminative to always obtain the expected microbial diversity during the iterative
569 processing of alignments. The coverage bias was calculated by obtaining fold-change
570 (Log_2) of species-specific read counting against the expected (theoretical) average for the
571 entire community according to information provided by the manufacturers.
572
573 *Illumina data analysis*
574 Fastq paired-end raw data (ENA experiment accession ERX2062322) was were assembled
575 using *Flash* software [27]. The HM782D and D6305 reads were de-multiplexed and
576 barcode and primers were removed using *Mothur* v1.36.1 [28]. The sequences were then
577 processed for chimera removal using *Uchime* algorithm [29] and SILVA reference set of
578 16S sequences [30]. A normalized subset of 10,000 sequences per sample was created by
579 random selection after shuffling (10,000X) of the original dataset. Taxonomy assessment
580 was performed using the RDP classifier v2.7 [31]. The Operational Taxonomic Unit
581 (OTU)-picking approach was performed with the normalized subset of 10,000 sequences
582 and the *uclust* algorithm implemented in USEARCH v8.0.1623 and the options -
583 *otu_radius_pct* equal 3 for clustering at 97% and -*min*size 2 for remove singletons [25].
584 SINA server was used for taxonomy identification of OTUs recovered from Illumina
585 MiSeq data [32].
586

Availability of supporting data

Accessions for the *rrn* database containing the reference sequences for alignments and taxonomic annotation is available at https://github.com/alfbenpa/rrn_db. The code source of the original *split_barcodes.pl* perl script is available at https://github.com/nanoporetech/barcoding/releases/tag/1.0.0 with ONT copyright.

Abbreviations

EC , European Commission; ENA, European Nucleotide Archive; HDF, Hierarchical Data Format; ITS, internal transcribed spacer; NCBI, National Center for Biotechnology Information; ONT, Oxford Nanopore Technologies; PCR, Polymerase Chain Reaction; rDNA, DNA encoding for the Ribosomal RNA; rRNA, Ribosomal RNA;*rrn*, the DNA region containing the 16S and 23S bacterial rRNA genes and its respective ITS region; SINA, SILVA Incremental Aligner; USB, Universal Serial Bus.

Competing interests

ABP is part of the MinION™ Access Programme (MAP).

Authors' contributions

ABP and YS designed the study and managed the project. ABP performed the experiments, analysed and managed the data. ABP draft the manuscript. Both authors read and approved the final manuscript.

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612

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Figure legends

Figure 1. Organization of the *rrn* region in bacteria. A - hypothetical transcriptional arrangements expected for *rrn* and tested experimentally using two sets of primer pairs (see small arrows drawn in each configuration). B - Agarose gel electrophoresis of PCR reactions performed under the two hypothetical arrangements of *rrn*; lanes: 1) 1kb ruler (Fermentas), 2) PCR reaction from the top configuration in panel A, 3) PCR reaction from the bottom configuration in panel A. The GelAnalyser Java application was used to perform the band size analysis of the 1kb ruler standard (C) and the amplicons obtained from human faecal DNA (D).

Figure 2. Variability of the *rrn* region and its functional domains. The *rrn* database compiled after parsing more than 67,000 draft and complete bacterial genomes was assessed by clustering analysis at different levels of sequence identity: 97 (white bars), 98 (light grey bars), 99 (dark grey bars), and 100% (black bars). For comparative aims, the functional DNA sequences encoded into the *rrn* region were also individually studied. The normalized diversity (y axis) resulted from calculate the number of clusters obtained for each analysis normalized with the median sizes of respective regions in terms of kb, and referenced against the value obtained for 16S sequences clustered at 97%, the canonical threshold for species assignment.

Figure 3. Microbial structure of the mock communities. A and B - microbial species and respective relative proportions determined to be present in the HM782D and D6305 mock communities, respectively, following the analysis of raw data obtained from *rrn* amplicon sequencing in the MinION^{TM} and chemistry R9. C and D - Comparative analysis of the expected microbial species and proportions against the data obtained after mapping of reads generated by a 4.5kbp amplicon PCR and sequenced in MinION^{TM} device with R9 and R9.4 chemistries, for HM782D and D6305 respectively. E - Linear correlation analysis of relative read proportions obtained for all bacterial species present in HM872D and D6305 mock communities with R9 and R9.4 chemistries.

Supplementary Material 1. Comparison of MinION™ and MiSeq outputs. Data obtained from Illumina MiSeq sequencing of V4-V5 16S region from respective mock communities was compared with outputs from MinION™ R9 and R9.4. Given that taxonomy identification of MiSeq reads at species level only retrieved very few assignments, we compiled the species distribution of MinION™ data at genus level.

Supplementary Material 2. MS Excel file compiling the output information retrieved from SINA server (https://www.arb-silva.de/aligner/) for taxonomy identification of 41 OTUs derived from analysis of HM782D with Illumina MiSeq approach. Information regarding sequence quality, identity percentage, mapping coordinates against *E. coli* reference, length and taxonomy based on SILVA, Greengenes, and RDP databases is showed for all OTU.

Supplementary Material 3. MS Excel file compiling the output information retrieved from SINA server (https://www.arb-silva.de/aligner/) for taxonomy identification of 18

760 OTUs derived from analysis of D6305 with Illumina MiSeq approach. Information
761 regarding sequence quality, identity percentage, mapping coordinates against *E. coli*
762 reference, length and taxonomy based on SILVA, Greengenes, and RDP databases is
763 showed for all OTU.
764

Table 1. Barcodes and primers used to generate amplicon libraries.

Sample	Barcode	Primer	Barcode extended[1]
HM-782D	(bc01) GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCT	(S-D-Bact-0008-c-S-20) AGAGTTTGATCMTGGCTCAG	(bc01F) GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCTAGAGTTTGATCMTGGCTCAG
	(bc01) GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCT	(23S-2241R) ACCGCCCCAGTHAAACT	(bc01R) GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCTACCGCCCCAGTHAAACT
D6503	(bc08) GGTGCTGTTCAGGGAACAAACCAAGTTACGTTTAACCT	(S-D-Bact-0008-c-S-20) AGAGTTTGATCMTGGCTCAG	(bc08F) GGTGCTGTTCAGGGAACAAACCAAGTTACGTTTAACCTAGAGTTTGATCMTGGCTCAG
	(bc08) GGTGCTGTTCAGGGAACAAACCAAGTTACGTTTAACCT	(23S-2241R) ACCGCCCCAGTHAAACT	(bc08R) GGTGCTGTTCAGGGAACAAACCAAGTTACGTTTAACCTACCGCCCCAGTHAAACT
Other primers used			
Human fecal DNA		(S-D-Bact-1391-a-A-17) GACGGGCGGTGWGTRCA	
		(23S-129F) CYGAATGGGRVAACC	

1 Underlined sequences correspond with the barcode sequence

Table 2. Basic stats comparison of R9 and R9.4 reads after processing.

	R9[1]	**R9.4**[1]
Total raw data	17,038 (100%)	39,216 (100%)
Reads > 1.5kb	12,278 (72%)	33,764 (86%)
Read length distribution	25th percentile = 2,847nt Median = 3,303nt 75th percentile = 3,754	25th percentile = 3,730nt Median = 3,976nt 75th percentile = 4,135nt
Reads aligned (bc1 + bc8)	3,227 (19%)	14,392 (43%)
Alignment identity distribution	25th percentile = 66.5% Median = 69 % 75th percentile = 73%	25th percentile = 81% Median = 85% 75th percentile = 87%
Maximum identity	86.7%	92%

1 The experiment and run accessions for the R9 data at ENA are ERX1676087 and ERR1605520, respectively.

2 The experiment and run accessions for R9.4 data at ENA are ERX1981944 and ERR1924217, respectively.

Table 3. Comparative analysis among data generated from MinION and MiSeq platforms.

	Relative read proportion				Coverage bias		
Genera HM782D	**Reference**	**PCR+MiSeq**	**PCR+MinION-R9**	**PCR+MinION-R9.4**	**PCR+MiSeq**	**PCR+MinION-R9**	**PCR+MinION-R9.4**
Acinetobacter spp.	0.050	0.019	0.046	0.032	-1.42	-0.12	-0.62
Actinomyces spp.	0.050	0.010	0.001	0.003	-2.36	-5.36	-4.02
Bacillus spp.	0.050	0.017	0.102	0.045	-1.57	1.03	-0.16
Bacteroides spp.	0.050	0.106	0.059	0.037	1.08	0.25	-0.42
Clostridium spp.	0.050	0.125	0.027	0.032	1.32	-0.91	-0.66
Deinococcus spp.	0.050	0.109	0.000	0.000	1.12	ND	ND
Enterococcus spp.	0.050	0.022	0.012	0.013	-1.17	-2.04	-1.93
Escherichia/Shigella spp.	0.050	0.038	0.172	0.209	-0.38	1.79	2.07
Helicobacter spp.	0.050	0.040	0.000	0.000	-0.32	ND	ND
Lactobacillus spp.	0.050	0.065	0.051	0.068	0.38	0.03	0.45
Listeria spp.	0.050	0.024	0.074	0.133	-1.04	0.57	1.41
Neisseria spp.	0.050	0.099	0.064	0.056	0.98	0.36	0.17
Propionibacterium spp.	0.050	0.021	0.079	0.097	-1.25	0.66	0.96
Pseudomonas spp.	0.050	0.038	0.018	0.079	-0.39	-1.46	0.67
Rhodobacter spp.	0.050	0.013	0.002	0.004	-1.91	-4.36	-3.51
Staphylococcus spp.	0.100	0.037	0.125	0.086	-1.44	0.32	-0.22
Streptococcus spp.	0.150	0.217	0.115	0.093	0.53	-0.38	-0.69
Genera D6305							
Bacillus spp.	0.139	0.574	0.383	0.340	2.04	1.46	1.29
Enterococcus spp.	0.088	0.078	0.057	0.082	-0.17	-0.64	-0.11
Escherichia/Shigella spp.	0.113	0.035	0.167	0.137	-1.67	0.56	0.28
Lactobacillus spp.	0.198	0.104	0.049	0.049	-0.93	-2.01	-2.01
Listeria spp.	0.163	0.046	0.080	0.118	-1.82	-1.03	-0.46
Pseudomonas spp.	0.058	0.060	0.038	0.039	0.05	-0.62	-0.56
Salmonella spp.	0.115	0.049	0.099	0.138	-1.22	-0.22	0.26
Staphylococcus spp.	0.126	0.051	0.094	0.095	-1.30	-0.42	-0.41
Average	**0.080**	**0.080**	**0.077**	**0.079**	**-0.51**	**-0.55**	**-0.36**
Median	**0.050**	**0.048**	**0.062**	**0.074**	**-0.72**	**-0.30**	**-0.29**
Min	**0.050**	**0.010**	**0.000**	**0.000**	**-2.36**	**-5.36**	**-4.02**
Max	**0.198**	**0.574**	**0.383**	**0.340**	**2.04**	**1.79**	**2.07**
Pearson's *r* [a] (p-value)	--	**0.39 (0.0504)**	**0.43 (0.0306)**	**0.41 (0.0417)**			
Pearson's *r* [b] (p-value)	--	**--**	**0.73 (0.0001)**	**0.64 (0.0005)**			

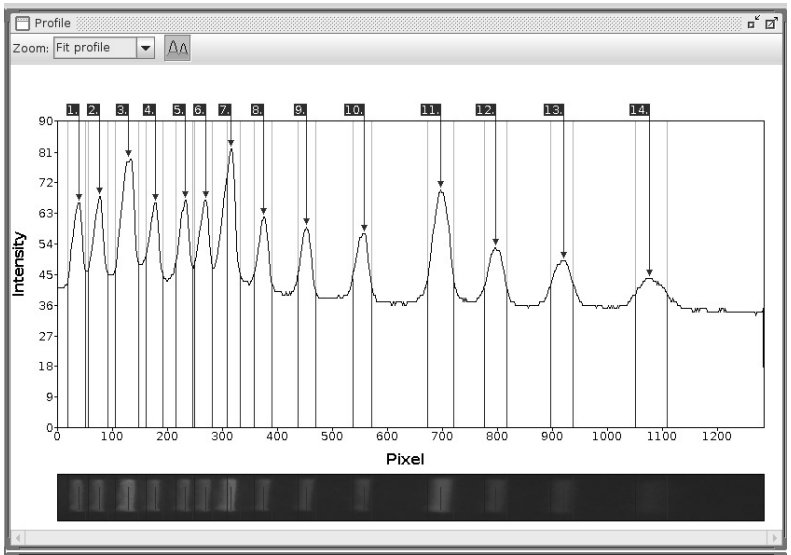
a Pearson's r calculated from comparisons of R9, R9.4, and MiSeq data with reference proportions, respectively.

b Pearson's r calculated from comparisons of R9 and R9.4 data with MiSeq output, respectively.

A

B

C

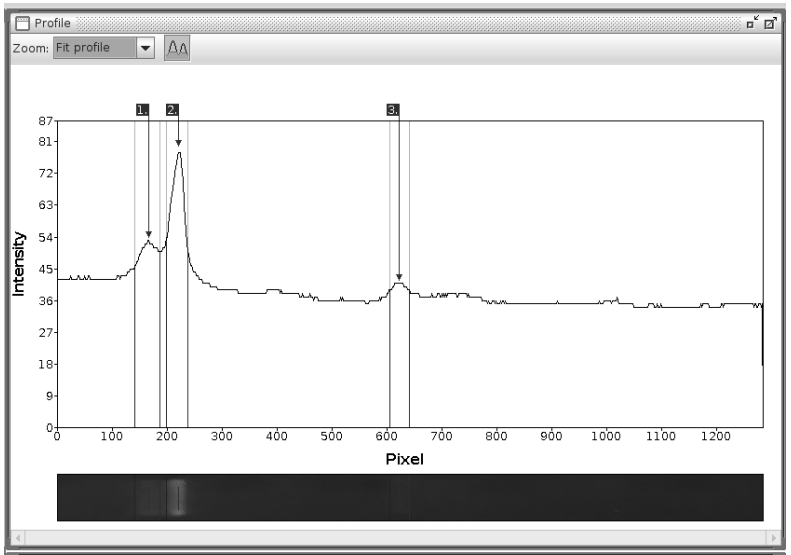


Analysis info

Show

Lane #	Band #	Rf	Raw volume	Cal. volume	MW
1.	1.	0.03	1895	-	**10000**
1.	2.	0.06	2041	-	**8000**
1.	3.	0.1	2802	-	**6000**
1.	4.	0.139	1775	-	**5000**
1.	5.	0.181	1794	-	**4000**
1.	6.	0.209	1906	-	**3500**
1.	7.	0.246	1739	-	**3000**
1.	8.	0.292	1768	-	**2500**
1.	9.	0.352	1698	-	**2000**
1.	10.	0.433	1803	-	**1500**
1.	11.	0.542	2840	-	**1000**
1.	12.	0.619	2030	-	**750**
1.	13.	0.716	1913	-	**500**
1.	14.	0.837	2489	-	**250**

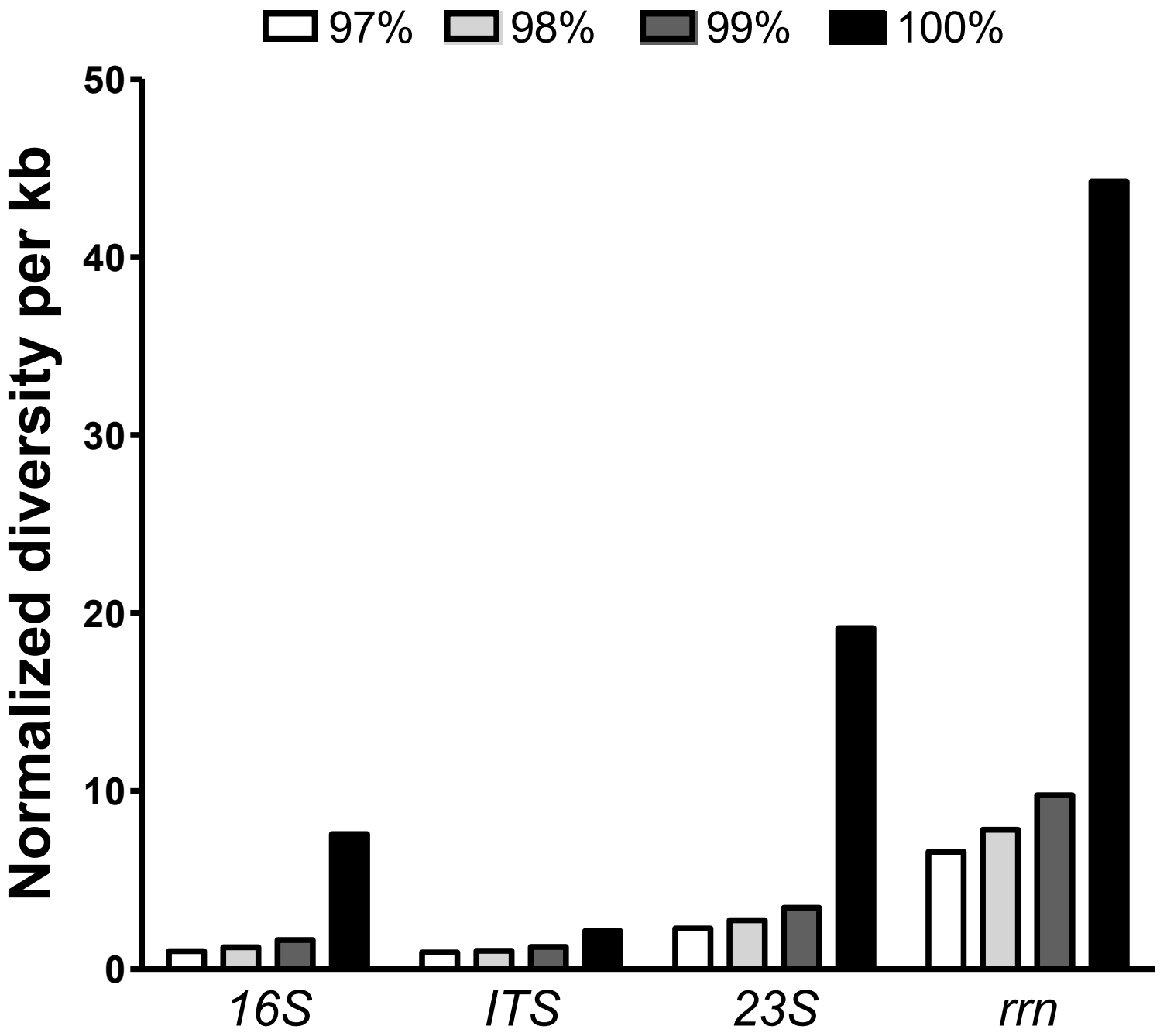
D



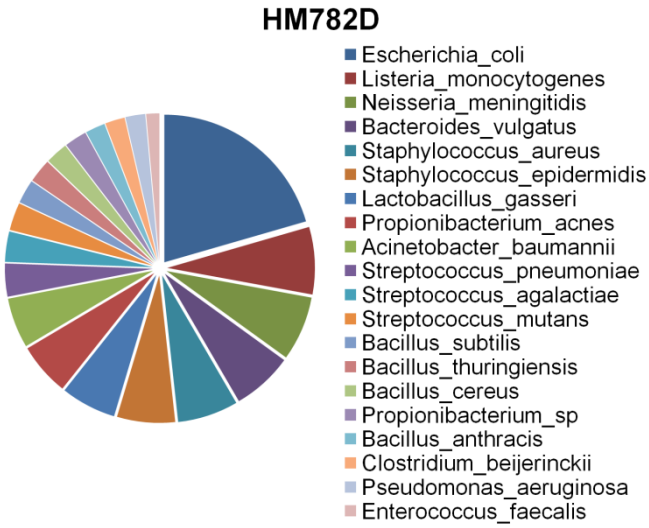
Analysis info

Show

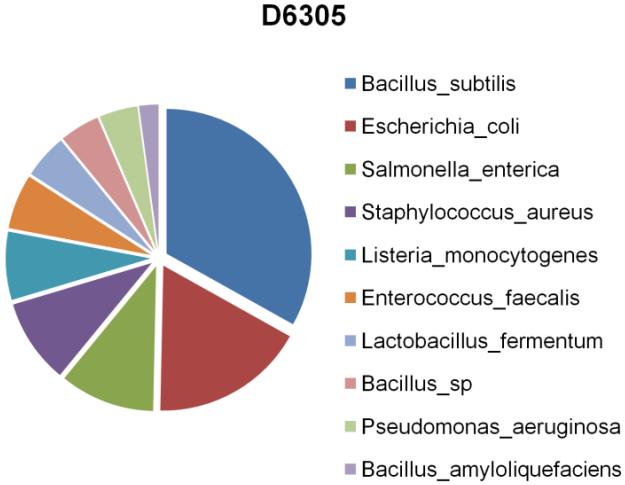
Lane #	Band #	Rf	Raw volume	Cal. volume	MW
2.	1.	0.129	2315	-	5441
2.	2.	0.171	2602	-	4304
2.	3.	0.484	1488	-	1061


97%
98%
99%
100%
Normalized diversity per kb
50
40
30
20
10
0
16S
ITS
23S
rrn

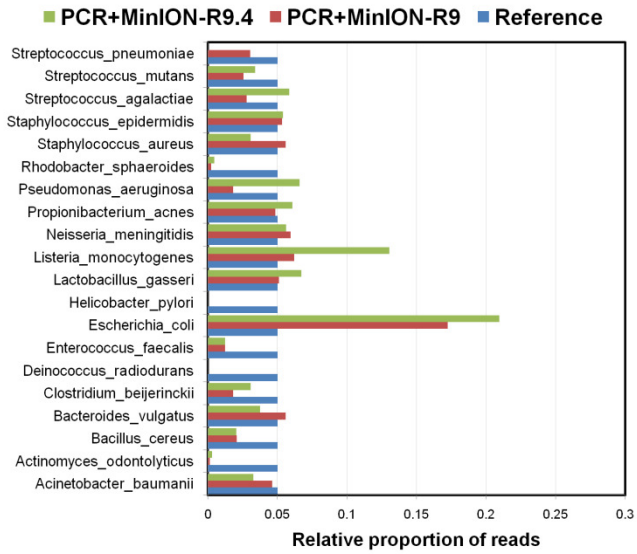
A



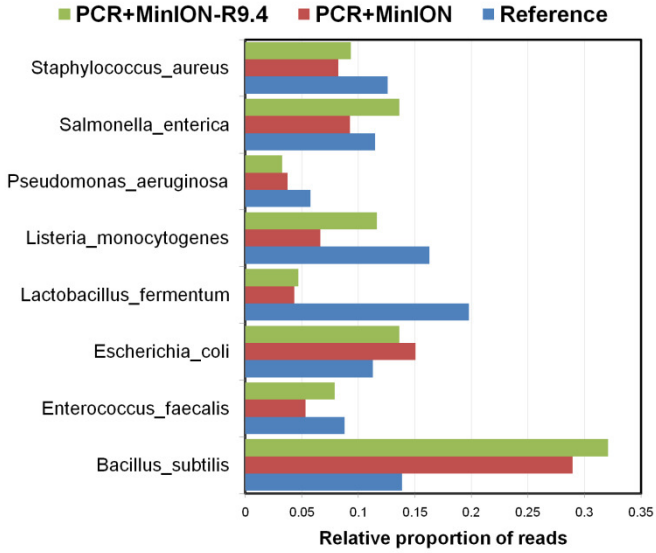
B



C



D



E