Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinION™ nanopore sequencing confers species-level resolution

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Abstract

Background: Species-level genetic characterization of complex bacterial communities has important clinical applications in both diagnosis and treatment. Amplicon sequencing of the 16S ribosomal RNA (rRNA) gene has proven to be a powerful strategy for the taxonomic classification of bacteria. This study aims to improve the method for full-length 16S rRNA gene analysis using the nanopore long-read sequencer MinION™. We compared it to the conventional short-read sequencing method in both a mock bacterial community and human fecal samples.

Results: We modified our existing protocol for full-length 16S amplicon sequencing by MinION™. A new strategy for library construction with an optimized primer set overcame PCR-associated bias and enabled taxonomic classification across a broad range of bacterial species. We compared the performance of full-length and short-read 16S amplicon sequencing for the characterization of human gut microbiota with a complex bacterial composition. The relative abundance of dominant bacterial genera was highly similar between full-length and short-read sequencing. At the species level, MinION™ long-read sequencing had better resolution for discriminating between members of particular taxa such as *Bifidobacterium*, allowing an accurate representation of the sample bacterial composition.

Conclusions: Our present microbiome study, comparing the discriminatory power of full-length and short-read sequencing, clearly illustrated the analytical advantage of sequencing the full-length 16S rRNA gene, which provided the requisite species-level resolution and accuracy in clinical settings.

Keywords:

16S rRNA, gut microbiota, MinION™, nanopore sequencing

Background

Recent advances in DNA sequencing technology have had a revolutionary impact on clinical microbiology [1]. Next-generation sequencing (NGS) technology enables parallel sequencing of DNA on a massive scale to generate vast quantities of accurate data. NGS platforms are now increasingly used in the field of clinical research [2]. Metagenomic sequencing offers numerous advantages over traditional culture-based techniques that have long been the standard test for detecting pathogenic bacteria. This method is particularly useful for characterizing uncultured bacteria and novel pathogens [3].

Among sequence-based bacterial analyses, amplicon sequencing of the 16S ribosomal RNA (rRNA) gene has proven to be a reliable and efficient option for taxonomic classification [4, 5]. The bacterial 16S rRNA gene contains nine variable regions (V1 to V9) that are separated by highly conserved sequences across different taxa. For bacterial identification, the 16S rRNA gene is first amplified by polymerase chain reaction (PCR) with primers annealing to conserved regions and then sequenced. The sequencing data are subjected to bioinformatic analysis in which the variable regions are used to discriminate between bacterial taxa [6].

Since the conventional parallel-type short-read sequencer cannot yield reads covering the full length of the 16S rRNA gene [7], several regions of it have been targeted for sequencing, which often causes ambiguity in taxonomic classification [8]. New sequencing platforms have overcome these technical restrictions, particularly those affecting read length. A prime example is the MinION™ sequencer from Oxford Nanopore Technologies, which is capable of producing long sequences with no theoretical read length limit [9-11]. MinION™ sequencing targets the entire 16S rRNA gene, allowing the identification of bacteria with more accuracy and sensitivity [12, 13]. Furthermore, MinION™ produces sequencing data in real time, which reduces turnaround time for data processing [14, 15].

Given these features of MinION™ sequencing, we had previously conducted full-length 16S amplicon sequencing analyses using the MinION™ platform coupled to a bioinformatics pipeline, which allowed us to identify bacterial pathogens with a total analysis time of under two hours [16]. However, we also found that the approach of using the commercial 16S Barcoding Kit (SQK-RAB204) available from Oxford Nanopore Technologies has a limited ability to detect particular taxa such as *Bifidobacterium* [16]. This is probably due to sequence mismatches in the primer used for 16S gene amplification [17]. Deviations or aberrancies in the *Bifidobacterium* composition in the human gut have been reported in several diseases including obesity, allergy, and inflammatory disorders [18]. Based on their putative health-promoting effects, several strains of *Bifidobacterium* have been utilized as probiotics [19]. Within these contexts, the species-level characterization of *Bifidobacterium* diversity in human gut microbiota is potentially important in clinical practice.

Our 16S rRNA gene sequence analysis using MinION™ has been tested only with pre-characterized mock bacterial DNA and a limited number of pathogenic bacteria from a patient-derived sample [20]. Its applicability to highly complex bacterial communities has not yet been thoroughly investigated. Therefore, in this study we modified our existing protocol for 16S amplicon sequencing by MinION™ and applied it to human gut microbiota with a complex bacterial composition [21], including *Bifidobacterium*, to determine whether full-length 16S rRNA gene sequencing with MinION™ is an effective characterization tool.

Results

Classification of the mock bacterial community

The 16S rRNA gene sequence of *Bifidobacterium* has three base mismatches with the 27F forward primer provided in the commercial sequencing kit (16S Barcoding Kit, SQK-RAB204, Oxford Nanopore Technologies; Additional File 2: Supplementary Fig. S1a), which biases amplification toward underrepresentation of *Bifidobacterium* species (Additional File 2: Supplementary Fig S2, Additional File 3: Supplementary Table S1-S3). To overcome this drawback, we introduced three degenerate bases to the 16S rRNA gene-specific sequences of the primer (Additional File 2: Supplementary Fig. S1b). The competence of the modified primer set was then evaluated by 16S rRNA gene sequence analysis of a ten-species mock community. The V1-V9 region of the 16S rRNA gene was amplified by the four-primer PCR method with the rapid adapter attachment chemistry and sequenced (Fig. 1a). MinION™ sequencing generated 8651 pass reads (Table 1). Following adapter trimming and size selection, 6972 reads (80.6% of pass reads with an average lead length of 1473 bp) were retained for bacterial identification. Full-length 16S amplicon sequencing with the modified primer set led to the successful identification of all the ten bacterial genera, including *Bifidobacterium* (Fig. 1b, Additional File 3: Supplementary Table S4). At the species level, 92.5% of analyzed reads were correctly assigned to each bacterial taxon included in the mock community, demonstrating the excellent discriminatory power of this full-length sequencing method for species identification (Fig. 1c). The only exception was *Bacillus cereus*. Discrimination of *Bacillus cereus* from the closely related species such as *Bacillus anthracis* and *Bacillus thuringiensis* was not fully achieved with the 16S gene analysis using MinION™ (Additional File 4).

We compared the resolution of full-length and short-read 16S amplicon sequencing for the taxonomic classification of bacteria. The V3-V4 region was amplified by four-primer PCR from the ten-species mock community DNA, and the samples were

138 sequenced on MinION™. After removing the adapter/barcode sequences and filtering
139 reads by length, 96189 reads with an average length of 454.9 bp for downstream
140 analysis were yielded (Table 1). In contrast to full-length sequencing with the highest
141 resolution, a significant number of V3-V4 reads could not be classified down to the
142 genus level, but could only be assigned to a higher taxonomic rank (Fig. 1b, 1c). Most
143 reads derived from *Enterococcus faecalis* and *Escherichia coli* were not assigned down
144 to each taxon, as more than two species produced the same similarity score for the V3-
145 V4 sequence read queries (Additional File 5). Such reads were classified to the parent
146 taxon, as more specific classification was impossible (Additional File 3: Supplementary
147 Table S5). The classifications were not affected by increasing the number of analyzed
148 reads to 10000 (Additional File 2: Supplementary Fig. S3, Additional File 3:
149 Supplementary Table S6).
150 For eight of the ten bacterial strains constituting the mock community, each subset of
151 V1-V9 sequencing reads classified to the specific genus were assigned with almost
152 complete accuracy (98.2-100%) to the corresponding species (Fig. 2). V3-V4 short-read
153 sequencing showed a discriminatory power comparable to that of V1-V9 full-length
154 sequencing in the classification of three genera (*Deinococcus*, *Rhodobacter*, and
155 *Streptococcus*) with more than 98% of reads correctly assigned. However, the V3-V4
156 region was not suitable for species-level identification of other taxa, such as *Clostridium*
157 and *Staphylococcus*. Only 0.2% of the V3-V4 reads belonging to the genus *Clostridium*
158 were assigned to *Clostridium beijerinckii*, a component of the mock community. In
159 contrast, 92.3% of *Clostridium* reads obtained from V1-V9 full-length sequencing were
160 correctly classified at the species level. These results suggest a lower resolution of the
161 V3-V4 region for species-level classification, emphasizing the advantage of long-read
162 sequencing for obtaining an accurate representation of the sample bacterial composition.
163
164 **Classification of human fecal bacteria**

165 We assessed the performance of our full-length 16S amplicon sequencing approach in
166 the context of a highly complex bacterial community. The V1-V9 region was amplified
167 by four-primer PCR from six human fecal samples (F1-F6) and analyzed by MinION™
168 sequencing. (Table 2). In Fig. 3, the numbers of species detected are plotted against the
169 numbers of reads analyzed. The curve started to plateau at around 20000 reads. There
170 was a highly significant correlation between the read numbers 20000 and 30000
171 (Pearson's correlation coefficient $r > 0.999$, Additional File 6: Supplementary Table S7).
172 Based on these observations, randomly sampled 20000 reads were used in further
173 analysis to determine the bacterial composition of the human gut.
174 For comparison, amplicon sequencing of the V3-V4 region was also conducted using
175 the MinION™ (Table 2) and the Illumina MiSeq™ platform (Table 3). The processed
176 reads from each data set were allocated to the reference bacterial genome using our
177 bioinformatics pipeline to determine the bacterial compositions (Additional File 7 for
178 V1-V9 MinION™ sequencing, Additional File 8 for V3-V4 MinION™ sequencing, and
179 Additional File 9 for V3-V4 MiSeq™ sequencing). From MiSeq™ sequencing data, the
180 bacterial composition was also analyzed by the operational taxonomic unit (OTU)-
181 based approach using the QIIME 2 (ver. 2019.7) pipeline (Additional File 2:
182 Supplementary Fig. S4, Additional File 10) [22, 23]. Although *Bacteroides* was
183 underrepresented in the OTU-based analysis, the two analytical methods (our
184 bioinformatics pipeline and OTU-based method) produced similar taxonomic profiles in
185 the dominant phylotypes for the MiSeq™ data. This result confirmed the validity of our
186 method for the taxonomic classification of the bacterial community.
187 The three sequencing methods (V1-V9 MinION™ sequencing, V3-V4 MinION™
188 sequencing, and V3-V4 MiSeq™ sequencing) revealed similar profiles for the six fecal
189 samples at the genus level (Fig. 4). Statistically significant similarities have been found
190 in the relative genus abundances across these sequencing methods. Thus, at the genus
191 level, V1-V9 full-length MinION™ sequencing exhibited a discriminatory power

192 comparable to that of high-quality short-read sequencing with MiSeq™ technology.

193

194 **The species-level taxonomic resolution achieved by full-length sequencing of the**

195 **16S rRNA gene using MinION™**

196 While genus classification using long versus short reads was relatively comparable, we

197 observed considerable differences across amplified regions in the species-level profiling

198 of human gut microbiota. As shown in Fig. 5, the number of ambiguous reads that were

199 not assigned to species but could be classified at a higher level was significantly greater

200 in the V3-V4 data set in comparison than in the V1-V9 data set.

201 When species compositions of the dominant taxa (*Bifidobacterium*, *Blautia*, and

202 *Bacteroides*) were analyzed, the V1-V9 and V3-V4 sequencing produced comparable

203 results for *Blautia* (Additional File 2: Supplementary Fig. S5, Additional File 6:

204 Supplementary Table S8) and *Bacteroides* genus (Additional File 2: Supplementary Fig.

205 S6, Additional File 6: Supplementary Table S9) in most of the fecal samples. For

206 *Bifidobacterium*, there appeared to be considerable deviations in the relative abundances

207 of some species depending on the sequencing method used (Fig. 6, Additional File 6:

208 Supplementary Table S10). Notably, most of the *Bifidobacterium* reads generated by

209 V1-V9 MinION™ sequencing were classified into the *Bifidobacterium* species that

210 were isolated from human sources [18, 24]. A significant number of the V3-V4 reads,

211 however, were assigned to *Bifidobacterium* species of non-human origin (Additional

212 File 2: Supplementary Fig. S7). From V3-V4 MiSeq™ sequencing data, the OTU-based

213 classification analysis using the QIIME 2 pipeline also revealed a lower resolution of

214 short-read sequencing for taxonomic separation of *Bifidobacterium* genus. Except for

215 *Bifidobacterium longum*, *Bifidobacterium* species could not be reliably identified by the

216 V3-V4 sequencing strategy (Additional File 2: Supplementary Fig. S8). These results

217 suggest that species classification of *Bifidobacterium* based on V3-V4 sequencing can

218 potentially lead to misidentification and biased community profiling that lacks

219 biological significance.

220

221

Discussion

16S rRNA amplicon sequencing is a powerful strategy for taxonomic classification of bacteria and has been extensively employed for analyzing samples from environmental and clinical sources [5, 25, 26]. We assessed the performance of MinION™ sequencing by comparing the resolution of the V1-V9 and V3-V4 reads for the taxonomic classification of bacteria. Due to the error-prone nature of MinION™ sequencing, the existing OTU-based approach, requiring at least 97% sequence identity threshold, has been considered unsuitable for taxonomic classification of MinION™ reads [27, 28]. Instead, the reads were analyzed by the direct read mapping method that assigns sequences to taxonomic bins based on the similarity to a reference database [14, 15]. Long-read MinION™ sequencing with the optimized primer set successfully identified *Bifidobacterium* species leading to a better representation of the species composition of the mock community. For improving the classification results, the reads were filtered by length to eliminate those outside the expected size range. Typically, extremely short reads possess only one primer-binding site, suggesting that they are derived from incomplete sequencing. There also exist unexpectedly longer reads with a continuous sequence structure in which two 16S amplicons are linked end-to-end. Because these reads can potentially result in unclassified reads or misclassification, they were eliminated before alignment to the reference sequences of the bacterial genome.

We also modified library construction for MinION™ sequencing with a four-primer PCR strategy, which enabled ligase-free adapter attachment to occur in a single-step reaction. The four-primer PCR generates amplicons with particular chemical modifications at the 5' ends to which adapter molecules can be attached non-enzymatically. Unlike the ligation-based approach, the PCR products amplified by the four-primer method are subjected directly to the adapter attachment reaction without repairing their 5' ends, substantially reducing the time required for sample preparation. Furthermore, because the protocol is free of Nanopore's transposase-based technology

249 (e.g. Rapid Sequencing Kit, SQK-RAD004) that cleaves DNA molecules to produce
250 chemically modified ends for library construction, the PCR products are kept intact,
251 enabling sequencing of the entire amplified region. Thus, the four-primer PCR-based
252 method allowed us to perform amplicon sequencing on the MinION™ platform with
253 user-defined arbitrary primer pairs, taking advantage of the rapid adapter attachment
254 chemistry. This method can be applied to a wide range of sequence-based analyses,
255 including detection of functional genetic markers like antimicrobial resistance genes
256 and identification of genetic variations in targeted loci [11, 29, 30].
257 Our present microbiome study, comparing the discriminatory power of the V1-V9 and
258 V3-V4 reads sequenced on the MinION™ platform, clearly illustrated the advantage of
259 sequencing the entire 16S rRNA gene. The full-length 16S gene sequencing provided
260 better resolution than short-read sequencing for discriminating between members of
261 certain bacterial taxa, including *Clostridium*, *Enterococcus*, *Escherichia*, and
262 *Staphylococcus*. Consistently, comprehensive *in silico* experiments using sequencing a
263 sequencing data set consisting of different regions of the 16S rRNA gene have shown
264 that the choice of the regions to be sequenced substantially affects the classification
265 results [6, 31]. As shown here and in previous publications, short-read sequencing of the
266 16S rRNA gene may be a reasonable option for providing a rough estimation of
267 bacterial diversity. However, it was not suited for analysis requiring species-level
268 resolution and accuracy, which was afforded by sequencing the entire 16S rRNA gene.
269 It is important to note that even full-length 16S gene analysis fails to discriminate some
270 closely related species such as members of *Bacillus cereus* group, indicating the
271 limitations of the 16S amplicon sequencing alone in species allocation. Long read
272 sequencing targeting other phylogenetic markers may be an alternative to 16S rRNA
273 amplicon sequencing and provide better resolution for bacterial identification.
274 In the analysis of the human fecal samples, we used the taxonomic resolution of the V3-
275 V4 region sequenced with MiSeq™, which generates highly accurate reads, as a

benchmark for the taxonomic resolution of the full-length 16S gene sequenced with MinION™. The relative abundance of dominant bacterial taxa was highly similar at the genus level between full-length MinION™ and short-read MiSeq™ sequencing. Despite the lower read quality, the full-length sequencing by MinION™ enabled reliable identification of bacterial genera with an accuracy comparable to MiSeq™ technology. The MiSeq™ platform enables 16S rRNA gene sequencing on a massive scale with reduced cost (approximately 20 USD per sample). Considering a low equipment price (1000 USD) and an affordable per-run cost (approximately 50 USD per sample), the MinION™ sequencer could be a viable option for practical applications in clinical microbiology.

At the species level, MinION™ long-read sequencing had better resolution for accurate identification of the composition of human gut microbiota. Composition profiles of *Bifidobacterium*, one of the dominant genera present in the human gut [32], appeared to differ considerably between the two sequencing platforms. While most MinION™ V1-V9 reads were assigned to *Bifidobacterium* species of human origin, a significant number of the MiSeq™ V3-V4 reads were assigned to non-human *Bifidobacterium* species [24]. Such improbable errors in species classification may be attributed to the lower resolution provided by the V3-V4 region, but in fact, 16S rRNA gene sequence analysis of mouse gut microbiota has revealed that V3-V4 reads sequenced on MiSeq™ are not well-suited for classifying *Bifidobacterium* species, consistent with our findings [33]. Since the taxonomic assignment is a critical step for analyzing the bacterial diversity and community composition, the reference database quality and the alignment algorithm must be further evaluated for each sequencing data set.

Conclusions

Our modified protocol for 16S amplicon sequencing overcame known limitations, such as the primer-associated bias toward the underrepresentation of *Bifidobacterium*, and

enabled taxonomic classification across a broad range of bacterial species. Benchmarking with MiSeq™ sequencing technology demonstrated the analytical advantage of sequencing the full-length 16S gene with MinION™, which provided the requisite species-level resolution and accuracy. With the recent progress in nanopore sequencing chemistry and base-calling algorithms, sequencing accuracy is continuously improving [34, 35]. This will soon enable us to exploit the full potential of MinION™ long-read sequencing technology. High-quality long sequences will allow better discrimination between closely related species, and even bacterial strains, in sequence-based bacterial analyses.

313 **Methods**

314 **Mock bacterial community DNA**

315 A mixture of bacterial DNA (10 Strain Even Mix Genomic Material, MSA-1000) was
316 obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA),
317 comprising genomic DNA prepared from the following ten bacterial strains: *Bacillus*
318 *cereus* (ATCC 10987), *Bifidobacterium adolescentis* (ATCC 15703), *Clostridium*
319 *beijerinckii* (ATCC 35702), *Deinococcus radiodurans* (ATCC BAA-816), *Enterococcus*
320 *faecalis* (ATCC 47077), *Escherichia coli* (ATCC 700926), *Lactobacillus gasseri* (ATCC
321 33323), *Rhodobacter sphaeroides* (ATCC 17029), *Staphylococcus epidermidis* (ATCC
322 12228), and *Streptococcus mutans* (ATCC 700610).

323

324 **Fecal DNA**

325 DNA was extracted from six human fecal samples using the NucleoSpin® Microbial
326 DNA Kit (Macherey-Nagel, Düren, Germany), as described previously [36]. Briefly,
327 human feces stored using the Feces Collection Kit (Techno Suruga Lab, Shizuoka,
328 Japan) were subjected to mechanical disruption by bead-beating, and DNA was isolated
329 using silica membrane spin columns. Extracted DNA was purified with the Agencourt
330 AMPure® XP (Beckman Coulter, Brea, CA, USA).

331

332 **16S rRNA gene sequencing on the MinION™ platform**

333 Four-primer PCR with rapid adapter attachment chemistry generated 16S gene
334 amplicons with modified 5′ ends for simplified post-PCR adapter attachment following
335 the manufacturer's instructions with slight modifications. For amplification of the V1-
336 V9 region of the 16S rRNA gene, the following inner primers were used, with 16S
337 rRNA gene-specific sequences underlined: forward primer (S-D-Bact-0008-c-S-20 [37])
338 with anchor sequence 5′-
339 TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG-3′ and reverse

340 primer (1492R) with anchor sequence 5′-
341 ACTTGCCTGTCGCTCTATCTTCCGGYTACCTTGTTACGACTT-3′. For
342 amplification of the V3-V4 region, the following inner primers were used, with 16S
343 rRNA gene-specific sequences underlined: 341F with anchor sequence 5′-
344 TTTCTGTTGGTGCTGATATTGCCCTACGGGNGGCWGCAG-3′ and 806R with
345 anchor sequence 5′-
346 ACTTGCCTGTCGCTCTATCTTCGGACTACHVGGGTWTCTAAT-3′. PCR
347 amplification of 16S rRNA genes was conducted using the KAPA2G™ Robust HotStart
348 ReadyMix PCR Kit (Kapa Biosystems, Wilmington, MA, USA) in a total volume of 25
349 µl containing inner primer pairs (50 nM each) and the barcoded outer primer mixture
350 (3%) from the PCR Barcoding Kit (SQK-PBK004; Oxford Nanopore Technologies,
351 Oxford, UK). Amplification was performed with the following PCR conditions: initial
352 denaturation at 95 °C for 3 min, 5 cycles of 95 °C for 15 sec, 55 °C for 15 sec, and
353 72 °C for 30 sec, 30 cycles of 95 °C for 15 sec, 62 °C for 15 sec, and 72 °C for 30 sec,
354 followed by a final extension at 72 °C for 1 min. Amplified DNA was purified using
355 AMPure® XP (Beckman Coulter) and quantified by a NanoDrop® 1000 (Thermo
356 Fischer Scientific, Waltham, MA, USA). A total of 100 ng of DNA was incubated with
357 1 µl of Rapid Adapter at room temperature for 5 min. The prepared DNA library (11 µl)
358 was mixed with 34 µl of Sequencing Buffer, 25.5 µl of Loading Beads, and 4.5 µl of
359 water, loaded onto the R9.4 flow cell (FLO-MIN106; Oxford Nanopore Technologies),
360 and sequenced on the MinION™ Mk1B. MINKNOW software ver. 1.11.5 (Oxford
361 Nanopore Technologies) was used for data acquisition.
362
363 **16S rRNA gene sequencing on the MiSeq™ platform**
364 Sequencing libraries were constructed as described previously [36]. Briefly, the V3-V4
365 regions of the 16S rRNA gene were amplified using a 16S (V3–V4) Metagenomic
366 Library Construction Kit for NGS (Takara Bio Inc, Kusatsu, Japan). The following

367 primers were used (16S rRNA gene-specific sequences are underlined): 341F with
368 overhang adapter 5′-
369 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-
370 3′ and 806R with overhang adapter 5′-
371 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCT
372 AAT-3′. The second PCR was performed using the Nextera® XT Index Kit (Illumina,
373 San Diego, CA, USA) for sample multiplexing with index adapters. The libraries were
374 sequenced on the MiSeq™ platform using the MiSeq™ Reagent Kit v3 (2 × 250 bp;
375 Illumina).
376
377 **Bioinformatics analysis**
378 Albacore software ver. 2.3.4 (Oxford Nanopore Technologies) was used for basecalling
379 the MinION™ sequencing data (FAST5 files) to generate pass reads (FASTQ format)
380 with a mean quality score > 7. The adapter and barcode sequences were trimmed using
381 the EPI2ME Fastq Barcoding workflow ver. 3.10.4 (Oxford Nanopore Technologies).
382 The reads were filtered by size using SeqKit software ver. 0.10.0 [38], retaining 1300-
383 1950 bp sequences for the V1-V9 region and 350-600 bp sequences for the V3-V4
384 region, based on the size distribution of 16S rRNA gene sequences in the SILVA
385 database ver. 132 [39, 40]. The average Phred quality score was assessed using
386 NanoPlot ver. 1.27.0 [41]. The processed reads from each set were analyzed using our
387 bioinformatics pipeline [42], as described previously [14, 15]. Briefly, FASTQ files
388 were converted to FASTA files. Simple repetitive sequences were masked using the
389 TANTAN program ver. 18 with default parameters [43]. To remove reads derived from
390 human DNA, we searched each read against the human genome (GRCh38) using
391 minimap2 ver. 2.14 with a map-ont-option [44]. Then, unmatched reads were regarded
392 as reads derived from bacteria. For each read, a minimap2 search with 5850
393 representative bacterial genome sequences stored in the GenomeSync database

394 (Additional File 1) [45] was performed. For each read, the species showing the highest
395 minimap2 score were assigned to the query sequence. When more than two species
396 showed the same similarity score, the reads were classified at any higher taxonomic
397 rank covering all the identified species. Taxa were determined based on the NCBI
398 taxonomy database [46]. Low-abundance taxa with less than 0.01% of total reads were
399 discarded from the analysis.
400
401 **Statistical analyses**
402 Differences between groups were evaluated by one-way analysis of variance (ANOVA)
403 followed by Dunnett's test for multiple comparisons. The Pearson correlation
404 coefficient was computed to compare the bacterial compositions analyzed by different
405 sequencing methods. Statistical significance was defined by a P-value < 0.05. Statistical
406 analyses were performed with Prism8 (GraphPad Software, Inc. La Jolla, CA, USA).
407
408 **Statement of ethics**
409 The Sunkaky Institutional Review Board approved this study (No. 2017-27). All
410 participants provided written informed consent.
411

412 **Abbreviations**

413 NGS: next-generation sequencing; OTU: operational taxonomic unit; PCR: polymerase
414 chain reaction; rRNA: ribosomal RNA

415

416 **Additional files**

417 **Additional File 1:** Representative bacterial genomes stored in the GenomeSync
418 database.

419 **Additional File 2: Fig. S1.** Sequence heterogeneities of the 27F primer-annealing site
420 in 16S rRNA genes. **Fig. S2.** Evaluation of 16S rRNA PCR primers for identification of
421 bacterial species. **Fig. S3.** Effect of read number on taxonomic classification. **Fig. S4.**
422 Rarefaction curves of observed OTUs in 16S V3-V4 amplicon sequencing of human
423 fecal samples using the MiSeq™ platform. **Fig. S5.** Species composition of *Blautia* in
424 human fecal samples. **Fig. S6.** Species composition of *Bacteroides* in human fecal
425 samples. **Fig. S7.** Deviations in the relative abundances of *Bifidobacterium* species in
426 human fecal samples. **Fig. S8.** Comparison of species composition of fecal
427 *Bifidobacterium* between classification methods.

428 **Additional File 3: Tables S1-S6.** Taxonomic assignment of the mock community
429 analyzed by MinION™ sequencing.

430 **Additional File 4:** Alignment search results for V1-V9 amplicon sequencing of the
431 mock community.

432 **Additional File 5:** Alignment search results for V3-V4 amplicon sequencing of the
433 mock community.

434 **Additional File 6: Table S7.** Correlations between numbers of reads and numbers of
435 detected species in 16S rRNA gene sequencing of human fecal samples. **Table S8.**
436 Comparison of species composition of fecal *Blautia* between sequencing methods.
437 **Table S9.** Comparison of species composition of fecal *Bacteroides* between sequencing
438 methods. **Table S10.** Comparison of species composition of fecal *Bifidobacterium*

between sequencing methods.

Additional File 7: Taxonomic profile of human fecal samples from MinION™ sequencing (amplicons: V1-V9).

Additional File 8: Taxonomic profile of human fecal samples from MinION™ sequencing (amplicons: V3-V4).

Additional File 9: Taxonomic profile of human fecal samples from MiSeq™ sequencing (amplicons: V3-V4).

Additional File 10: Taxonomic profiles of human fecal samples from MiSeq™ sequencing (amplicons: V3-V4, taxonomic classification by OTU-based analysis using the QIIME 2 pipeline).

Declarations

Ethics approval and consent to participate

This study was approved by the Sunkaky Institutional Review Board (No. 2017-27).

Consent for publication

All participants provided written informed consent.

Availability of data and materials

The sequence datasets supporting the conclusions of this article are available in the DDBJ DRA database (https://www.ddbj.nig.ac.jp/dra/index-e.html) under accession numbers DRR225043 to DRR225065.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YMa, KK, AF, YMo, YN, SN and KH designed and supervised the study. SK, AF, YMo, and HO contributed to sample collection. YMa, KM, and TT conducted the experiments. YMa, YYasumizu, YYasuoka, and SN analyzed the data. YMa wrote the manuscript. YYasumizu, HB, SN, and KH contributed to editing the manuscript. All authors read and approved the final manuscript.

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608 **Figure legends**

609 **Fig. 1** 16S rRNA gene sequence analysis using the MinION™ nanopore sequencer. **a**
610 Workflow of 16S rRNA amplicon sequencing on the MinION™ platform. Sequencing
611 libraries are generated by the four-primer PCR-based strategy, enabling simplified post-
612 PCR adapter attachment. At the initial stage of PCR, the 16S rRNA gene is amplified
613 with the inner primer pairs. The resulting PCR products are targeted for amplification
614 with the outer primers to introduce the barcode and tag sequences at both ends, to which
615 adapter molecules can be attached in a single-step reaction. **b, c** Taxonomic assignments
616 of a mock community analyzed by MinION™ sequencing. The V1-V9 or V3-V4 region
617 of the 16S rRNA gene was amplified from a pre-characterized mock community sample
618 comprising ten bacterial species and sequenced on the MinION™ platform. Three
619 thousand reads were randomly selected from the processed data set and aligned directly
620 to the reference genome database of 5850 representative bacterial species. The pie
621 charts represent taxonomic profiles at the (b) genus and (c) species levels. Slices
622 corresponding to misclassified (assigned to bacteria not present in the mock
623 community) or unclassified (not classified at the species level but placed in a higher
624 taxonomic rank) reads are exploded. The relative abundance (%) of each taxon is
625 shown.

626

627 **Fig. 2** Accurate taxonomic assignment afforded by full-length MinION™ sequencing of
628 the 16S rRNA gene. Classification accuracy compared between full-length (V1-V9) and
629 partial (V3-V4) 16S sequencing data obtained from composition profiling of the ten-
630 species mock community. The donut charts show the proportions of reads correctly
631 assigned to the species constituting the mock community. The percentage of correctly
632 classified reads is shown in the center hole. NA: not assigned (no reads were classified
633 in *Escherichia* genus).

634

635 **Fig. 3** 16S rRNA gene sequence analysis of human gut microbiota. Six human fecal
636 samples (F1-F6) were subjected to full-length 16S rRNA amplicon sequencing via
637 MinION™. Numbers of detected species are plotted against numbers of reads used for
638 taxonomic classification.
639
640 **Fig. 4** Comparison of taxonomic profiles of human gut microbiota between sequencing
641 methodologies. Six fecal samples (F1-F6) were analyzed by sequencing the entire 16S
642 rRNA gene using MinION™ (N_V1-V9). For comparison, the V3-V4 region was
643 sequenced on MinION™ (N_V3-V4) or MiSeq™ platforms (I_V1-V9). Randomly
644 sampled 20000 reads from each data set were allocated to the reference genome
645 database of 5850 representative bacterial species. A heat map shows the relative genus
646 abundance (%) of classified reads. The 15 most abundant taxa are shown. The Pearson
647 correlation coefficient (r) between sequencing methods was computed. Asterisks
648 indicate significant correlations at $P < 0.05$.
649
650 **Fig. 5** Comparison of taxonomic resolution. The percentages of ambiguous reads not
651 assigned to the species level are plotted for six fecal samples analyzed by MinION™
652 (N_V1-V9 and N_V3-V4) or MiSeq™ (I_V3-V4). Horizontal bars represent mean
653 values. * $P < 0.05$ (statistically significant).
654
655 **Fig. 6** Species composition of *Bifidobacterium* in six fecal samples. MinION™ V1-V9
656 sequencing confers species-level resolution for bacterial composition profiling. Results
657 obtained by the three sequencing methods are shown. The legends show the 14 most
658 abundant *Bifidobacterium* species.
659

Table 1 MinION™ sequencing statistics for the mock community sample

Primers	Pass reads				Trimmed reads		Filtered reads		
	No. of reads	Min (bp)	Avg (bp)	Max (bp)	No. of reads	Avg (bp)	No. of reads	Avg (bp)	Q score
V1-V9	8651	237	1497	3292	8455	1367.1	6972 (80.6%)	1473	9.0
V3-V4	101372	180	585.7	1977	99937	451.8	96189 (94.9%)	454.9	9.2

Min: minimum read length, Avg: average read length, Max: maximum read length, Q score: average Phred quality score. The percentage of reads retained after size filtering is shown in parentheses.

Table 2 Statistics of MinION™ sequencing data for human fecal samples

Sample	Pass reads				Trimmed reads		Filtered reads		
	No. of reads	Min (bp)	Avg (bp)	Max (bp)	No. of reads	Avg (bp)	No. of reads	Avg (bp)	Q score
F1/N V1-V9	104895	186	1521.1	4549	103100	1386.4	89752 (85.6%)	1463.7	9.4
F2/N V1-V9	84065	169	1393.8	4253	82458	1259.8	60326 (71.8%)	1461.4	9.4
F3/N V1-V9	76968	168	1474.3	4829	74479	1343.3	60,713 (78.9%)	1465.5	9.4
F4/N V1-V9	114060	168	1541.7	4836	111436	1410.6	100569 (88.2%)	1469.9	9.4
F5/N V1-V9	85912	177	1536.0	4877	83038	1405.4	74168 (86.3%)	1474.2	9.4
F6/N V1-V9	108938	213	1525.1	4866	106857	1393.5	93146 (85.5%)	1467.4	9.4
F1/N V3-V4	52864	160	568.8	2759	52283	435.2	48494 (91.7%)	442.5	9.1
F2/N V3-V4	92816	174	583.4	2886	91989	442.8	89016 (95.9%)	444.7	9.2
F3/N V3-V4	60200	163	568.5	2062	59435	434.6	55706 (92.5%)	441.1	9.2
F4/N V3-V4	83021	202	578.0	2050	81734	446.1	77995 (93.9%)	450.0	9.2
F5/N V3-V4	78409	167	578.4	1796	76135	447.8	72526 (92.5%)	453.1	9.2
F6/N V3-V4	74931	114	580.3	2246	73946	446.1	71330 (95.2%)	449.1	9.2

N: Oxford Nanopore MinION™, Min: minimum read length, Avg: average read length, Max: maximum read length, Q score: average Phred quality score. The percentage of reads retained after size filtering is shown in parentheses.

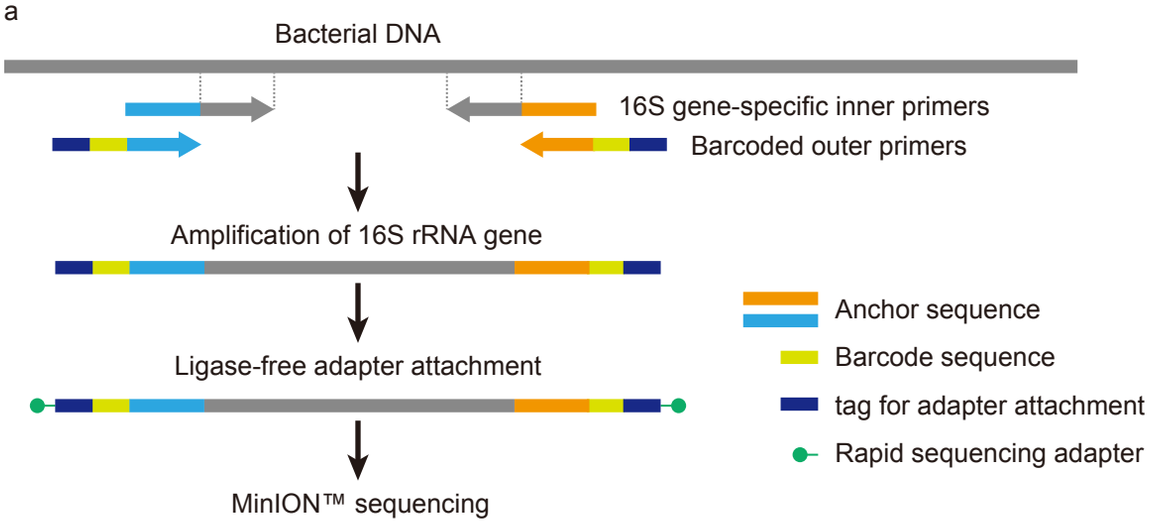
Table 3 Statistics of MiSeq™ sequencing data for human fecal samples

Sample	Paired reads	Merged reads		Filtered reads		
	No. of reads	No. of reads	Avg (bp)	No. of reads	Avg (bp)	Q Score
F1/I V3-V4	66242	63821	449.3	63778 (96.3%)	449.5	31.4
F2/I V3-V4	68824	66640	447.6	66490 (96.6%)	448.3	32.3
F3/I V3-V4	132057	128095	446.9	127999 (96.9%)	447.1	31.6
F4/I V3-V4	103532	100945	451.4	100853 (97.4%)	451.7	32.8
F5/I V3-V4	72136	70521	451	70459 (97.7%)	451.3	33.2
F6/I V3-V4	52182	50907	449	50841 (97.4%)	449.5	33.2

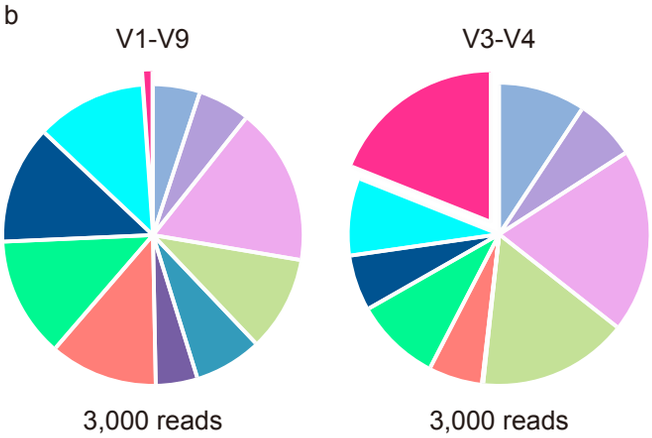
I: Illumina MiSeq™, Avg: average read length, Q score: average Phred quality score. The percentage of reads retained after size filtering is shown in parentheses.

Fig. 1

a

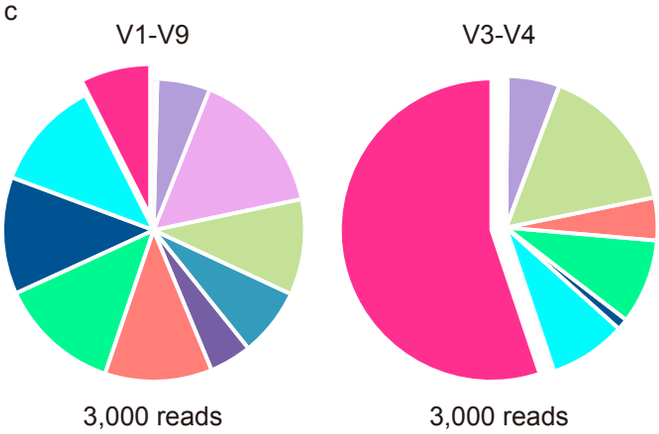


b



Genus	V1-V9	V3-V4
Bacillus	5.07%	9.30%
Bifidobacterium	5.63%	6.60%
Clostridium	16.97%	19.70%
Deinococcus	10.23%	16.10%
Enterococcus	7.30%	0.13%
Escherichia	4.50%	0.00%
Lactobacillus	11.63%	5.77%
Rhodobacter	12.97%	9.17%
Staphylococcus	12.73%	5.97%
Streptococcus	11.87%	8.30%
mis- or unclassified	1.10%	18.97%

c



Species	V1-V9	V3-V4
B. cereus	0.40%	0.17%
B. adolescentis	5.63%	5.53%
C. beijerinckii	15.67%	0.03%
D. radiodurans	10.23%	16.07%
E. faecalis	7.30%	0.00%
E. coli	4.50%	0.00%
L. gasseri	11.47%	4.57%
R. sphaeroides	12.97%	9.07%
S. epidermidis	12.50%	1.20%
S. mutans	11.87%	8.20%
mis- or unclassified	7.47%	55.17%

Fig. 2

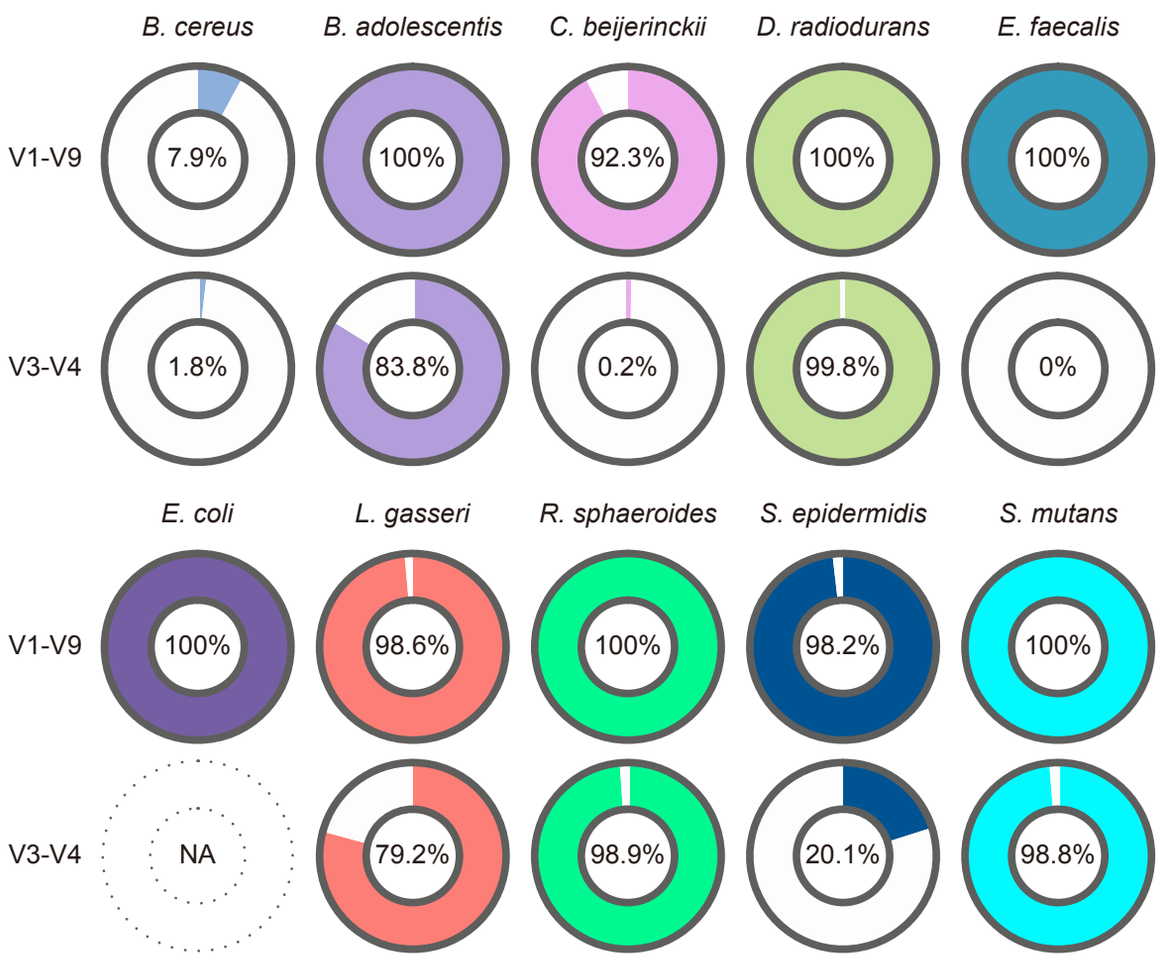

B. cereus
B. adolescentis
C. beijerinckii
D. radiodurans
E. faecalis
V1-V9
7.9%
100%
92.3%
100%
100%
V3-V4
1.8%
83.8%
0.2%
99.8%
0%
E. coli
L. gasseri
R. sphaeroides
S. epidermidis
S. mutans
V1-V9
100%
98.6%
100%
98.2%
100%
V3-V4
NA
79.2%
98.9%
20.1%
98.8%

Fig. 3

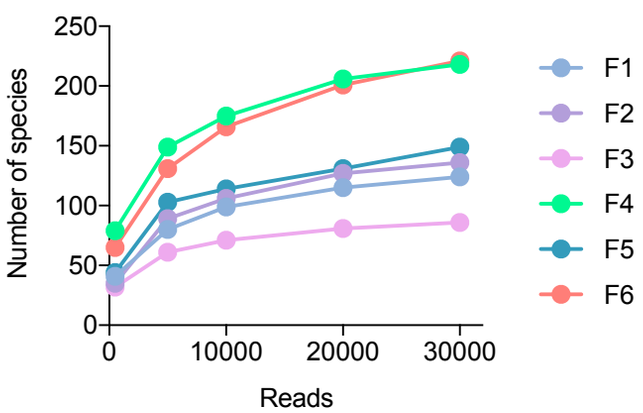

250
200
150
100
50
0
Number of species
0
10000
20000
30000
Reads
F1
F2
F3
F4
F5
F6

Fig. 4

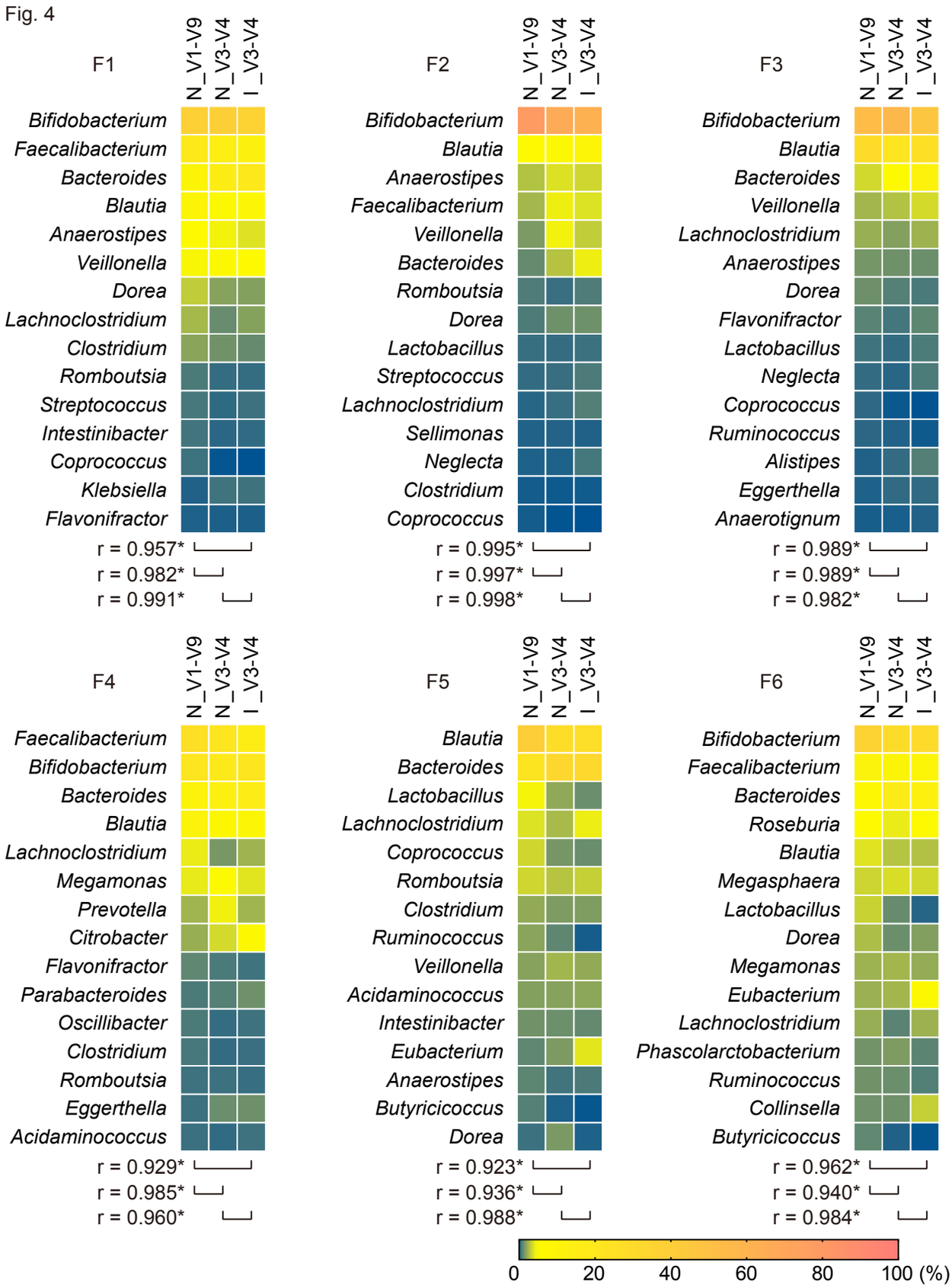

F1
N_V1-V9
N_V3-V4
I_V3-V4
Bifidobacterium
Faecalibacterium
Bacteroides
Blautia
Anaerostipes
Veillonella
Dorea
Lachnoclostridium
Clostridium
Romboutsia
Streptococcus
Intestinibacter
Coprococcus
Klebsiella
Flavonifractor
r = 0.957*
r = 0.982*
r = 0.991*
F2
N_V1-V9
N_V3-V4
I_V3-V4
Bifidobacterium
Blautia
Anaerostipes
Faecalibacterium
Veillonella
Bacteroides
Romboutsia
Dorea
Lactobacillus
Streptococcus
Lachnoclostridium
Sellimonas
Neglecta
Clostridium
Coprococcus
r = 0.995*
r = 0.997*
r = 0.998*
F3
N_V1-V9
N_V3-V4
I_V3-V4
Bifidobacterium
Blautia
Bacteroides
Veillonella
Lachnoclostridium
Anaerostipes
Dorea
Flavonifractor
Lactobacillus
Neglecta
Coprococcus
Ruminococcus
Alistipes
Eggerthella
Anaerotignum
r = 0.989*
r = 0.989*
r = 0.982*
F4
N_V1-V9
N_V3-V4
I_V3-V4
Faecalibacterium
Bifidobacterium
Bacteroides
Blautia
Lachnoclostridium
Megamonas
Prevotella
Citrobacter
Flavonifractor
Parabacteroides
Oscillibacter
Clostridium
Romboutsia
Eggerthella
Acidaminococcus
r = 0.929*
r = 0.985*
r = 0.960*
F5
N_V1-V9
N_V3-V4
I_V3-V4
Blautia
Bacteroides
Lactobacillus
Lachnoclostridium
Coprococcus
Romboutsia
Clostridium
Ruminococcus
Veillonella
Acidaminococcus
Intestinibacter
Eubacterium
Anaerostipes
Butyricicoccus
Dorea
r = 0.923*
r = 0.936*
r = 0.988*
F6
N_V1-V9
N_V3-V4
I_V3-V4
Bifidobacterium
Faecalibacterium
Bacteroides
Roseburia
Blautia
Megasphaera
Lactobacillus
Dorea
Megamonas
Eubacterium
Lachnoclostridium
Phascolarctobacterium
Ruminococcus
Collinsella
Butyricicoccus
r = 0.962*
r = 0.940*
r = 0.984*
0
20
40
60
80
100 (%)

Fig. 5

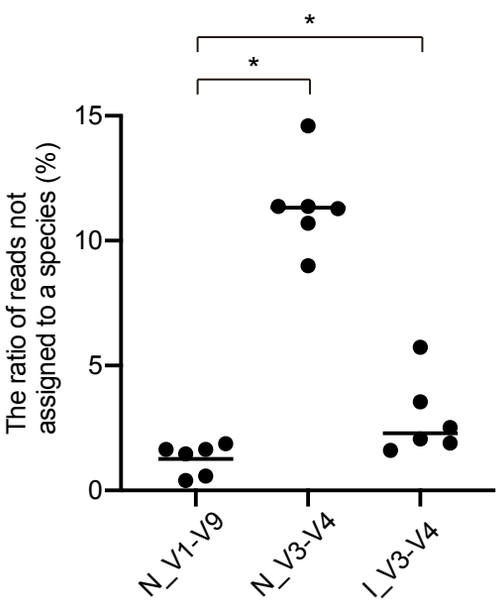
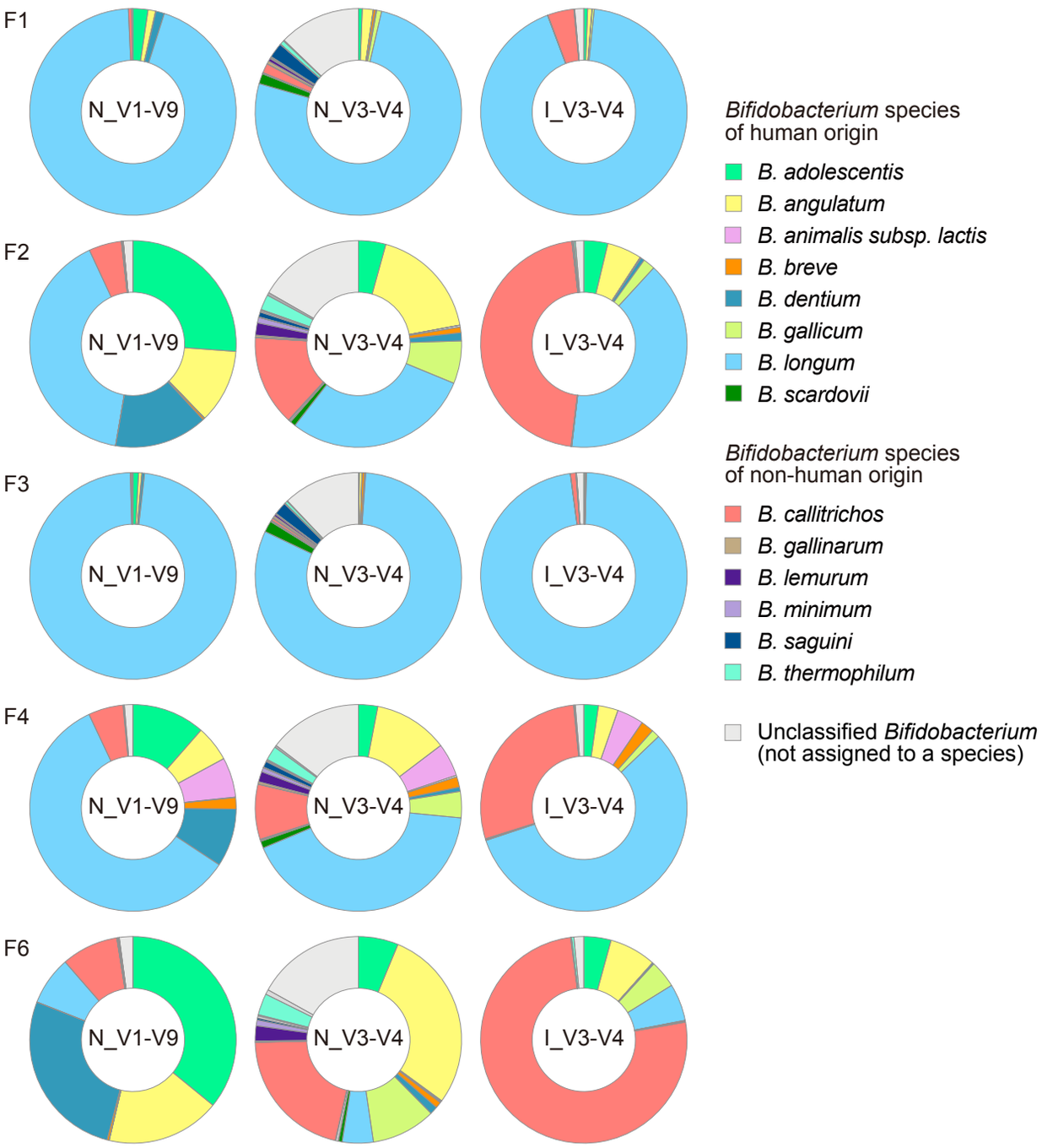

*
*
15
10
5
0
The ratio of reads not
assigned to a species (%)
N_V1-V9
N_V3-V4
I_V3-V4

Fig. 6

a


F1
F2
F3
F4
F6
N_V1-V9
N_V3-V4
I_V3-V4
Bifidobacterium species of human origin
B. adolescentis
B. angulatum
B. animalis subsp. lactis
B. breve
B. dentium
B. gallicum
B. longum
B. scardovii
Bifidobacterium species of non-human origin
B. callitrichos
B. gallinarum
B. lemurum
B. minimum
B. saguini
B. thermophilum
Unclassified Bifidobacterium (not assigned to a species)