Improved microbial community characterization of 16S rRNA via metagenome hybridization capture enrichment

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Keywords: amplicon, microbial diversity, microbiome, mock communities, next generation sequencing, shotgun libraries, target enrichment

Abstract

Environmental microbial diversity is often investigated from a molecular perspective using 16S ribosomal RNA (rRNA) gene amplicons and shotgun metagenomics. While amplicon methods are fast, low-cost, and have curated reference databases, they can suffer from amplification bias and are limited in genomic scope. In contrast, shotgun metagenomic methods sample more genomic regions with fewer sequence acquisition biases. However, shotgun metagenomic sequencing is much more expensive (even with moderate sequencing depth) and computationally challenging. Here, we

develop a set of 16S rRNA sequence capture baits that offer a potential middle ground with the advantages from both approaches for investigating microbial communities. These baits cover the diversity of all 16S rRNA sequences available in the Greengenes (v. 13.5) database, with no sequence having < 80% sequence similarity to at least one bait for all segments of 16S. The use of our baits provide comparable results to 16S amplicon libraries and shotgun metagenomic libraries when assigning taxonomic units from 16S sequences within the metagenomic reads. We demonstrate that 16S rRNA capture baits can be used on a range of microbial samples (i.e., mock communities and rodent fecal samples) to increase the proportion of 16S rRNA sequences (average >400-fold) and decrease analysis time to obtain consistent community assessments. Furthermore, our study reveals that bioinformatic methods used to analyze sequencing data may have a greater influence on estimates of community composition than library preparation method used, likely in part to the extent and curation of the reference databases considered.

1 Introduction

The study of microbes is critically important, as they have many essential roles in ecosystem function, disease pathology, host physiology, and possibly assessing infectious disease outbreaks (Dueker et al., 2018; Gallardo-Escárate et al., 2020). As microbial communities can often be highly diverse and complex, it can be challenging to identify rare taxa in complex environmental samples (e.g., soil, freshwater, etc.) with traditional and modern techniques (i.e., culturing, 16S amplicons, or metagenomic shotgun libraries). Advances in sequencing technologies have transformed traditional microbiology. Microbial communities that were previously considered indiscernible or unstudied, can now be investigated at greater depths than ever before from many different environmental systems (Gilmour et al., 2010; Kustin et al., 2019).

For decades, the 16S small subunit ribosomal RNA (rRNA) gene has been the gold standard marker for microbial molecular taxonomic research (Woese and Fox, 1977; Meola et al., 2015), as this highly conserved gene contains nine rapidly evolving hypervariable regions that aid in species identification (Yuan et al., 2015). Amplicon sequencing, targeting the 16S rRNA, is a cost-effective and high-throughput method used to study aquatic, terrestrial, food- and host-associated microbial communities (Logares et al., 2014; Polka et al., 2015; Jiang et al., 2016; Jousselin et al., 2016; Jouglin et al., 2019; Suenami et al., 2019; Ziegler et al., 2019). However, studies relying on 16S rRNA amplicon sequencing have limitations and biases. Relevant biases in 16S rRNA amplicon sequencing are associated with DNA extraction, amplification via PCR, sequencing, and sequence

analysis (Kennedy et al., 2014; Knight et al., 2018). Specifically, PCR biases include primer bias (Klindworth et al., 2013; Kelly et al., 2019) and varying GC content (Aird et al., 2011). Additional limitations associated with amplicon sequencing include challenges in the taxonomic characterization of microbial communities, as well as accuracy and availability of reference databases (Kennedy et al., 2014; Poretsky et al., 2014; Ritari et al., 2015; Knight et al., 2018). Furthermore, the selection of the hypervariable region used for the amplicon analysis (i.e., V1-V3; V3-V4; V4; etc) can lead to differences in bacterial identification (Vetrovsky and Baldrian, 2013; Martinez-Porchas et al., 2016).

In more recent years, metagenomic shotgun sequencing has aimed to characterize taxonomic profiles of unique clade-specific marker genes to provide a balanced view of community composition and function (Neelakanta and Sultana, 2013; Knight et al., 2018). However, metagenomic sequencing has its own limitations; genomic DNA may contain non-target DNA (e.g., human DNA), which can affect downstream analysis (e.g., mis-assemblies of sequence contigs, spurious reads) thus leading to inaccurate conclusions (Schmieder and Edwards, 2011; Gasc and Peyret, 2018). Also, metagenomic libraries are more expensive, take longer to prepare, and are much more complex than amplicon libraries, requiring more computational effort (Sekse et al., 2017). In particular, it is difficult to identify low abundance genetic traits and rare taxa using metagenomic libraries, and extensive deep sequencing is often required to do so (Lasa et al., 2019). In summary, shotgun sequencing is less biased and yields data on many genomic regions, but the main tradeoffs are high costs of library preparation, sequencing, and analysis.

Mock communities can be used to help establish ground truth in microbial diversity studies, in particular when comparing different library preparation methods (Costea et al., 2017; Rausch et al., 2019). Rausch et al., 2019 provided a comparison of 16S rRNA amplicon sequencing and metagenomic sequencing, and revealed similar community makeup (i.e., abundance and taxa diversity) of their shallow mock community regardless of library type. Conversely, other studies have found key differences in abundance and taxa of mock communites attributed to wet-laboratory methods (Costea et al., 2017; Rausch et al., 2019). However, some of these differences may be attributed to varying bioinformatic tactics.

In terms of bioinformatic analyses, advantages and limitation of methods, reference databases, and software have been vastly described for both 16S rRNA and metagenomic strategies (Truong et al., 2015; Callahan et al., 2016a; Costea et al., 2017; Escobar-Zepeda et al., 2018; Rausch et al., 2019). The variation among these can lead to a lack of sensitivity and specificity that may contribute to wrong classifications and/or no classification at a specific taxonomic level, and erroneous abundance

assignments (Escobar-Zepeda et al., 2018). In particular, it can be challenging to analyze environmental samples, as most reference databases are based on human commensals (Dueholm et al., 2020).

Both strategies (i.e., 16S rRNA amplicon and metagenomic shotgun libraries) present their own challenges and variations in analyses (Knight et al., 2018), but metagenomic shotgun libraries tend to perform at a higher sensitivity and specificity than 16S rRNA amplicon data (Escobar-Zepeda et al., 2018). For metagenomic data, programs like MetaPhlAn2 may be used to classify and estimate the relative abundance of microbial cells by mapping reads against marker sequences to classify the sequences at the sub-species to higher taxonomic levels (i.e., marker-gene approach) (Segata et al., 2012; Truong et al., 2015). Whereas 16S rRNA amplicon data is commonly analyzed by inferring representative sequences using a variety of methods, some of which are influenced by fragment size and 16S region (Edgar, 2013; Callahan et al., 2016a; Callahan et al., 2016b). Furthermore, some methods used to assign operational taxonomic units may result in limited resolution at lower taxonomic levels (e.g., genus and species levels), as even organisms that share 98.75% sequences may be different species (Mysara et al., 2017). Reference databases for 16S rRNA are much more extensive than those for metagenomic analyses, which is key for superior analysis, particuraly in environmental samples (Escobar-Zepeda et al., 2018). However, variation in taxonomic classification and abundance has also been associated with the use of different reference databases (Jovel et al., 2016; Rausch et al., 2019).

Hybridization capture (also known as sequence capture, target capture, or targeted sequence capture) is an enrichment technique that uses a set of biotinylated DNA or RNA baits that are complementary to DNA sequences of interest to increase the proportion of DNA fragments of interest within DNA libraries, subsequently characterizing the DNA by massively parallel sequencing (Lasa et al., 2019). Hybridization capture assays have been designed previously for the 16S rRNA gene, using 15-1,402 baits (Gasc and Peyret, 2018; Barrett et al., 2020). Additional hybridization capture bait sets have been designed for a variety of microbial projects, such as sets of defined pathogens or particular genes, including virulence genes for *Vibrio* spp. that infect oysters (Lasa et al., 2019), bifidobacterial in the gut of mammals (Lugli et al., 2019), and antibiotic resistance genes (Guitor et al., 2019). Importantly, unlike other culture independent techniques, hybridization capture provides greater phylogenetic resolution and increased sensitivity, while requiring fewer sequencing reads (Lasa et al., 2019; Barrett et al., 2020). More specifically, 16S rRNA capture baits provide a cost-effective way to identify bacteria in diverse environmental samples and identify rare taxa.

Here, we present a hybridization capture method (i.e., 16S-cap) to enrich metagenomic shotgun libraries for DNA sequences of 16S rRNA genes. Our protocol improves on the existing methods by including many more baits that better cover known sequence variation in 16S databases, taking advantage of the extensive reference databases and ease of analyses of 16S rRNA sequences for taxonomic classification and decreasing bias introduced from primer affinity, while reducing sequencing costs per sample compared to unenriched metagenomic libraries. For microbes, targeted sequence capture techniques for 16S rRNA have shown more accurate representation of microbial communities compared to traditional methods (i.e., 16S rRNA amplicons, shotgun libraries) (Gasc and Peyret, 2018). We provide a comparison of traditional methods for assessing composition of microbial communities (i.e., 16S rRNA amplicons and metagenomic shotgun libraries) with our 16S-cap method to characterize *in silico* mock, *in vitro* mock, and real microbial communities from genomic data.

2. Materials & Methods

2.1 Samples and DNA Extraction

We used two commercial standard genomic DNA mock community collections to characterize simple communities (HM-276D, BEI Resources, Manassas, VA; D6306, Zymo Research, Irvine, CA). For complex communities, we used a subset of fecal samples from previous studies that examined the impacts of environmental xenobiotic agents on the gut microbial communities of rodent models (Gao et al., 2017; Wang et al., 2018). The first study examined carbamate insecticide in male C57BL/6 mice (i.e., *Mus musculus*) (Gao et al., 2017), and the second examined green tea polyphenols in female Sprague-Dawley rats (i.e., *Rattus norvegicus*) (Wang et al., 2018). DNA was extracted using Qiagen Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) or PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). Details on experimental design and extractions are previously described (Gao et al., 2017; Wang et al., 2018).

2.2 16S rRNA Amplicon Metabarcoding

The primer pairs targeting the V3 and V4 16S regions (S-D- Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21) (Klindworth et al., 2013) were used for amplification of the 16S rRNA gene in rat fecal samples and mock communities; and the primer pair targeting the V4 region (515-F and 806-R) (Caporaso et al., 2012) was used on the mouse fecal samples. We created indexed fusion primers with TruSeq compatible sequencing oligos as previously described using the *Adapterama I* and

Adapterama II systems (Glenn et al., 2019a; Glenn et al., 2019b) to generate amplicon libraries using two rounds of PCR (Method 5 of Table 3 from Glenn et al. 2019b). For the first PCR, we prepared individual 25 µL PCR reactions for each sample using KAPA HiFi reagents (KAPA Biosystems, Wilmington, MA, USA). Each PCR reaction mix included 5 µL 5x KAPA HiFi buffer, 0.75 µL 10 mM dNTPs, 0.5 µL KAPA HiFi HotStart, 1.5 µL 5 µM forward indexed-fusion primer, 1.5 µL 5 µM reverse indexed-fusion primer, and 1 µL of 20 ng/µL DNA. PCR conditions were as follows: initial denaturation at 95°C for 3 min; 15-18 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec; final extension at 72°C for 5 min.

In preparation for the second PCR, we normalized individually indexed PCR products with a SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocols or by pooling them together based on agarose gel band brightness. These pools served as the template for a second limited cycle PCR. Each 25 µL PCR reaction mix included: 5 µL 5x KAPA HiFi buffer, 0.75 µL 10 mM dNTPs, 0.5 µL KAPA HiFi HotStart, 2.5 µL of 5 µM forward iTru5 primer, 2.5 µL of 5 µM reverse iTru7 primer, and 5 µL of product from the first PCR. The following were used as PCR conditions: initial denaturation at 95°C for 2 min; 10 cycles of 95°C for 20 sec, 60°C for 15 sec, and 72°C for 30 sec; final extension at 72°C for 5 min. These PCR products were purified with Sera-Mag magnetic beads (Thermo-Scientific, Waltham, MA, USA). We quantified the final products with a Qubit 2.0 Fluorometer (Thermo-Scientific, Waltham, MA, USA) and pooled them in equal molar ratios for sequencing. Samples were sequenced using an Illumina MiSeq v2 600 cycle kit (Illumina, San Diego, CA, USA) at the Georgia Genomics and Bioinformatics Core (Athens, GA, USA).

2.3 Metagenomic Libraries

Extracted DNA was sheared on a Bioruptor UCD-300 (Diagenode, Denville, NJ, USA) to an average size of about 500 bp. We input ~100 ng of fragmented DNA into each reaction of a KAPA HyperPrep Kit (KAPA Biosystems, Wilmington, MA, USA) following manufacturer's protocol at half volume reaction size with 14 PCR cycles using iTru adaptors and indexed primers (Glenn et al., 2019b). Samples were sequenced on an Illumina HiSeq 3000 with PE150 reads (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA).

2.4 16S rRNA Bait Design

We used Prokka v1.11 with default settings, to annotate and extract all 16S rRNA sequences in GreenGenes v13.5 to ensure that only 16S rRNA regions were represented in the final bait set (Seemann, 2014). Stretches of up to 25 Ns were replaced with T bases to facilitate probe design across short unknown regions. We then used USEARCH v8.1 (Edgar, 2010) to sort by length (large to short) and cluster (query coverage 90%, identity 90%) sequences, retaining one centroid from each cluster. We then designed 120mer baits with flexible ~50% overlap. These baits were then clustered using USEARCH (query coverage 75%, identity 78%), and one centroid per cluster retained.

2.5 16S rRNA Hybridization Capture Enrichments

Metagenomic libraries were combined into 500 ng pools of eight samples for rodents or two samples for mock communities. Target enrichments of each pool were performed using myBaits kit (Arbor Biosciences CAT # 308616, Ann Arbor, MI, USA) using the designed 16S rRNA Capture Baits following manufacturer's protocol (v3.01) with a 24-hour 65°C hybridization. Following hybridization, we used Dynabeads M-280 Streptavidin magnetic beads (Life Technologies, Carlsbad, CA, US) for capturing and washing each biotinyalted bait library. We then performed a post-enrichment amplification using Illumina P5/P7 primers (Illumina, San Diego, CA, USA) and KAPA HiFi HotStart reagents (KAPA Biosystems, Wilmington, MA, USA) using 98°C for 45 seconds, followed by 16-22 cycles of 98°C for 20 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, ending with a final extension of 72°C for five minutes. PCR products were cleaned 1:1 with Sera-Mag beads (Glenn et al., 2019a), quantified on Qubit and pooled in equimolar ratios for sequencing paired-end 150 bp and 300 bp reads on Illumina HiSeq 3000 (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) and MiSeq (Georgia Genomics Bioinformatics Core, Athens, GA, USA), respectively.

2.6 Simulating 16S rRNA Target Enrichment Data

Three metagenomes (i.e., Lindgreen synthetic metagenome (Lindgreen et al., 2016); Zymo Mock Community DS6306 genomes; and BEI Mock Community HM-276D) were used to simulate 16S rRNA capture data. In summary, a fasta file containing our 120mer bait set was mapped to each metagenome fasta file (Supplementary Data 1-3) using Burrows-Wheeler aligner (bwa) v.0.7.17 (Li and Durbin, 2009). Samtools v1.9 (Li et al., 2009) was used to convert the obtained sam file into a bam file. This mapping process is meant to simulate what would be an error- and bias-free hybridization process. Following this, we obtained the mapping coordinates of the baits on the reference metagenomes and extracted the sequences + 200 bp to the upstream and downstream of the

first position, if possible. Here, we sought to simulate a hybridization of the bait to the core of an ~500 bp fragment while obtaining the flanking regions typically captured from use of biotinylated baits.

The software ART 2016.06.05 (Huang et al., 2012) was then used to simulate > 200,000 paired-end 150 bp fastq reads from these extended reference sequences from each metagenome. These fastq files were mapped to Greengenes 97% similarity database v.13.8 using BBmap v. 38.50 (Bushnell, 2014). For each metagenome, we recorded the number of paired reads mapped to Greengenes, number of forward reads, number of reverse reads and percentage average total mapped, and compared these results with those from real samples also mapped to the Greengenes database (see below) (Altschul et al., 1990).

2.7 Data Processing and Analysis

After obtaining demultiplexed Illumina pair-end raw sequences, we used library specific pipelines to process the data (Figure 1). For 16S rRNA amplicon libraries, primers were removed using cutadapt v1.15 (Martin, 2011). Following this, DADA2 (v1.8) was used for quality trimming and filtering, de-replication and sequence-variant inference, merging paired-end reads, construction of feature tables, low relative abundance filtering of 0.5%, removal of chimeras, and taxonomy assignment (Callahan et al., 2016a). The taxonomy assignment was based on 97% clustered OUT based on Greengenes v13.8 database in the DADA2 pipeline.

[Insert Figure 1]

For 16S-cap libraries, the resulting quality filtered reads were mapped to the 97% clustered OTU based on Greengenes v13.8 database using BBmap v37.78 (Bushnell, 2014). The resulting mapping information was filtered, and a hit was recorded if both ends of paired read hit the same reference, or only one end of the paired read hit a reference. A low relative abundance filter of 0.5% was applied. Also, we assessed the presence of non-target reads in the quality-filtered dataset by 1) running MetaPhlAn2 v2.7.8 (Segata et al., 2012; Truong et al., 2015), and 2) mapping to the rat and mice genomes using Burrows-Wheeler aligner (bwa) v.0.7.17 (Li and Durbin, 2009).

For unenriched metagenomic libraries, Trimmomatic v0.36 (Bolger et al., 2014) was used for quality trimming using a sliding window of three nucleotides with an average Q > 20, and minimum length of 75 nucleotides. Reads that passed initial quality filtering (including both paired reads and orphan reads) were fed to MetaPhlAn2 v2.7.14 for taxonomy assignment (Segata et al., 2012; Truong et al., 2015). A low relative abundance filter of 0.5% was applied. To further compare to the results from

16S-cap analysis, we performed the same 16S mapping steps to the GreenGenes database as described for 16S-cap libraries for the unenriched libraries.

Data was analyzed using R statistical software (R Development Core Team, 2010). Duncan's multiple range test was used to compare abundance estimates between library types. Additionally, for all samples, abundance estimates were used to construct a Bray-Curtis dissimiarilty matrix, which was then used to generate a prinicle coordinate analysis (PCoA).

3 Results

3.1 16S rRNA Capture Bait Design

The 1,262,986 sequences comprising Greengenes v13.5 were annotated and 1,261,075 16S rRNA sequences were retained. A total of 117 sequences containing consecutive runs of 25 or more ambiguous bases (Ns) were removed. A total of 18,649 centroidal sequences were obtained from USEARCH clustering. From these sequences, 413,480 120mer baits were designed. These baits were then clustered using USEARCH, retaining one centroid per cluster, for a total of 37,745 baits.

3.2 Sequencing Summary Statistics

A summary of average sequence statistics for each sample and library preparation type is given in Table 1. For the 16S rRNA amplicon data, the number of total raw read pairs per sample ranged from 49,828 for the Zymo mock community to 136,184 for the BEI mock community, with rodent fecal samples having intermediate depth. More reads (~77%) remained from the rodent fecal samples after the denoising steps through the rigorous DADA2 pipelines versus the mock communities. Low percentages of high quality reads remained following filtering for both the BEI and Zymo mock communities (38.7% and 48.8% respectively). For the BEI mock community, initial index matching in R2 reads caused ~30% loss of data (versus less than 5% typically observed in other samples) and DADA2 quality trimming lost another ~30% of data. For the Zymo mock community, the loss of data was mainly due to chimeric filtering (~30% of data loss).

For the unenriched libraries, the highest number of total raw read pairs ranged from 4,985,957 in the Zymo mock community to 28,219,552 in the insecticide-treated mouse feces. The percentage of reads retained after filtering was greater than 65% for all unenriched libraries. The average percentage of reads mapped to GreenGenes ranged from 0.1% to 0.2% in the BEI and Zymo mock communities.

For 16S-cap libraries, the PE150 reads had higher numbers of reads on average per sample type than PE300 reads. The highest number of raw reads (i.e., 11,474,476) was obtained for the insecticide-treated mouse feces with PE150 reads. The percentage of reads after filtering were greater than 70% for all 16S-cap libraries. The average percentage of mapped reads was greater than 50% for all 16S-cap libraries, with the highest percentage of mapping in the 16S-cap BEI mock community sequenced with PE300 at 75.7%. On average among all sample types, the proportion of on target reads was increased 435-fold when compared to unenriched libraries (range 283 – 499 fold increase, Suppplemental Table 2).

[Insert Table 1]

3.3 16S rRNA Target Enrichment Simulated Reads

Summary information for simulated reads is given in Table 2. We observed a higher percentage of total mapped reads in our simulated mock communities than for the real data from those communities (Table 2). For example, the real data from the Zymo mock community had an average total mapping of 78.15% to GreenGenes, compared to 91.43% from the simulated data. Similarly, the BEI mock community had an average total mapping of 78.62% for the real data, compared to 92.37% for the simulated data.

[Insert Table 2]

3.4 Validation on Mock Community Samples

We initially prepared amplicon libraries, unenriched metagenomic libraries, and enriched our metagenomic libraries using the target enrichment bait set (i.e., 16S-cap) we developed using two mock communities (Table 1). At the phylum level both samples appear to provide accurate identification of the microbes with good estimates of abundance, regardless of library type or data analysis method used (Figure 2). The 16S-cap samples and metagenomic samples generate one detection of a false positive phyla in the mock community samples (Figure 2). Additionally, in both the unenriched and 16S-cap libraries analyzed with a 16S mapping approach, Cyanobacteria was found in low abundance even though it was not expected to be present in the mock community. However, when analyzing the unenriched library using marker gene approach, Cyanobacteria was not found and instead Ascomycota was identified.

[Insert Figure 2]

At the genus level, 16S-cap and unenriched libraries reflect more accurate microbial community composition and abundance for most taxa (Figure 3). The 16S-cap and unenriched libraries with 16S

mapping missed three genera: *Escherichia*, *Listeria*, and *Bacillus* for both mock community samples. However, after identifying presumably false positive genera with above 1% abundance in the samples analyzed with 16S mapping software, three families with no genus identification, *Enterobacteriaceae*, *Listeriaceae*, *Bacillaceae*, were found, suggesting these are likely the missing genera. In comparison, 16S rRNA amplicon-based analysis identified nearly all genera in mock samples, however, its estimates of abundance for *Actinomyces*, *Propionibacterium*, *Pseudomonas*, and *Rhodobacter* all greatly deviate from the nominal compositions. The unenriched metagenomic libraries analyzed with a marker-gene approach were able to identify all 18 genera in the mock communities, however its estimate of *Bacillus* abundance in both mock communities deviate from the nominal composition (Figure 3).

[Insert Figure 3]

[Insert Figure 4]

In the BEI mock community libraries, relative abundance estimates in the 16S-cap libraries were more accurate than the amplicon and unenriched libraries as measured by fold change being very close to 1 (Figure 4). In the amplicon library, several genera (i.e., *Pseudomonas*, *Actinomyces*, *Propionilbacterium*, and *Rhodobacter*) are beyond the 2-fold change of their nominal compositions. In particular one genus, *Rhodobacter*, proved to be challenging for all three library preparation methods for accurate estimation of relative abundance. Duncan's multiple range test revealed that there were significant differences (p-value ≤ 0.05) between the BEI mock community amplicon and 16S-cap libraries, whereas the unenriched libraries were not found to be significantly different than the amplicon or 16S-cap libraries. For the Zymo mock community libraries, relative abundance estimates in the 16S-cap libraries are more accurate than relative abundance estimates for the amplicon library. However, Duncan's multiple range test did not detect a significant difference between the three library types (i.e., amplicon, unenriched, and enriched) (Figure 4).

3.5 Validation on Fecal Samples

Principle coordinate analysis was performed on mock community samples and additional samples from laboratory mice and rats to further validate the 16S-cap method. When Bray-Curtis was used to construct the dissimilarity matrix, which considers abundance estimates, we found that regardless of analyses at the level of family (Figure 5A, left) or genus (Figure 5B, right) similar themes emerged. We observed that the mock community samples were similar to each other regardless of library type. Conversely, in the mouse and rat samples, we found that the unenriched libraries analyzed with a

marker-gene approach grouped together separately from amplicon, unenriched, and 16S-cap libraries, all of which were analyzed with the 16S mapping approach.

[Insert Figure 5]

A comparison of Bray-Curtis distance was performed for rodent fecal samples at the level of family and genus (Figure 6). This analysis revealed similar trends regardless of sample type or taxonomic rank. The 16S-cap and unenriched libraries analyzed with 16S mapping approach showed to be the most similar to each other, with a dissimilarity rate below 0.25. Bray-Curtis dissimilarity was higher when comparing the amplicon libraries to both 16S-cap and unenriched libraries. When comparing the unenriched libraries analyzed with two different analysis strategies (i.e., mapping reads to GreenGenes vs gene-marker approach), we observed the highest degree of dissimilarity at both the family and genus levels with dissimilarity rates at approximately 0.75. Post-hoc analysis revealed that there were significant differences when comparing the unenriched and 16S-cap libraries to all other library types, regardless of sample type or taxonomic rank (Figure 6).

[Insert Figure 6]

4 Discussion

Given the limitations of 16S rRNA amplicon and shotgun metagenomic libraries outlined in the introduction, we sought to provide an alternative method to identify microbial community composition by creating a 16S rRNA hybridization capture assay (i.e., 16S-cap). Our study revealed two important things: 1) our 16S-cap method is an efficient way to obtain sequences from the complete 16S rRNA gene to accurately reflect microbial community composition and abundance and 2) bioinformatic analysis methods greatly influence community composition in environmental samples, regardless of library type. In our study we observed that sequences from 16S-cap were not significantly different than sequences from unenriched shotgun libraries when analyzed using similar bioinformatic methods and databases. However, we did find that the 16S-cap assay requires far fewer reads, thus allowing enriched libraries to be characterized on benchtop sequencers, including Illumina MiSeq instruments, at reasonable cost while overcoming the previously mentioned limitations with direct 16S rRNA approaches and metagenomic approaches. These limitations include selection and drift bias in PCR during amplicon library preparation and the potential for non-target DNA (e.g., human DNA) in metagenomic libraries, which can lead to errors in downstream analyses.

Enrichment for genes of interest is an important technique in characterizing complex environmental samples. Previous studies have found other capture enrichment methods to increase the proportion of on target reads from ~0.1% in unenriched shotgun libraries to ~60% in enriched libraries (Gasc and Peyret, 2018). Similarly, we found 0.1 – 0.2% of unenriched libraries to map to the16S rRNA, whereas 58-76% of the enriched reads mapped to the 16S rRNA (Table 1). On average we achieved a 435-fold increase in reads mapped to the 16S rRNA in our 16S-cap libraries compared to the unenriched libraries (Supplementary Table 2). *In silico* simulations of 16S-cap revealed that under ideal conditions, 88-92% mapping to the 16S rRNA from mock communities could be achieved. Therefore, our 16S-cap enrichment process helps to achieve a very high percentage of on-target reads, but not quite as high as theoretically possible.

Our 16S-cap method identified several species that were not expected in the theoretical targets of the mock communities, which may be attributed to several factors. First, the lack of genus identification may be due to the mapping methods or clustering level used in data analysis rather than the library preparation method. Both the 16S-cap and unenriched libraries analyzed with a 16S mapping method failed to identify three genera *Escherichia*, *Listeria*, and some *Bacillus* in the mock communities. However, there are three familes, *Enterobacteriaceae*, *Listeriaceae*, and *Bacillaceae*, in the false positive genera with >1% abundance that align with our missing genera. Thus, it appears that reads for these three genera appear to be present, but are not being assigned appropriately at the genus level. By assigning these unidentified genera as *Escherichia*, *Listeria*, and *Bacillus* respectively, the 16S-cap library is highly accurate in terms of taxonomic classification and abundance. Taxonomic misassignement is a known problem with 16S mapping bioinformatic methods, and new software is in development (Schloss and Westcott, 2011; Pollock et al., 2018; Zinger et al., 2019; Djemiel et al., 2020). Additional work on the mapping and assignment processes used here, as well as comparisons of newly developed and commonly used bioinformatic software is beyond the scope of this paper, but warranted in future work. Other taxa identified that were not expected in the mock communities may be due to reagent contamination or index hopping during sequencing. Several studies show that contaminating DNA is common in laboratory reagents and DNA extraction kits (Salter et al., 2014; Weiss et al., 2014; Kim et al., 2017; Eisenhofer et al., 2019; Zinter et al., 2019). Furthermore, studies recommend sequencing negative controls consisting of ‘blank’ extractions and library preparations to identify contamination by bacterial species (Salter et al., 2014; Knight et al., 2018). Conversely, false positives of extremely low abundance (i.e., 0.1% or less) may be due to misassigned data that can

occur during Illumina sequencing. This phenomena is often referred to as index hopping (van der Valk et al., 2019).

We compared theoretical target values of the BEI resources and Zymo mock communities to all three library types (i.e., amplicon, unenriched, and 16S-cap) (Figures 3, 4). We find that the 16S-cap libraries are representative of the target abundance values of the mock communities (Figure 3). Post-hoc analysis revealed that the 16S rRNA amplicon library and 16S-cap library made from the BEI mock community were significantly different from each other (p-value ≤ 0.05) based on relative abundance. A PCoA revealed that in the mouse and rat samples the unenriched libraries analyzed with a marker-gene approach grouped together separately from 16S rRNA amplicon libraries and 16S-cap and unenriched libraries analyzed with taxonomic binning approach (Figure 5). Thus, enrichment and amplicon sequencing result in similar library composition, as do 16S-cap and unenriched libraries analyzed with a 16S taxonomic binning approach. This indicates that our 16S-cap method may be less biased than 16S amplification, but that analysis methods or the reference database may greatly influence community composition results. Walsh et al. (2018) analyzed different species classifiers using marker gene approaches and taxonomic binning, and found that the results of the marker gene approach (i.e., MetaPhlAn2) were different from taxonomic binning methods. Taxonomic binning methods are influenced by the size of the reference genome, whereas marker gene approaches are not (Droge and McHardy, 2012; Balvociute and Huson, 2017; Walsh et al., 2018). The use of hybridization capture baits may help alleviate some of these issues.

Other groups have designed a more limited bait set to hybridize all known 16S rRNA gene sequences by focusing on highly conserved regions and incorporating ambiguities(Gasc and Peyret, 2018). When validating their bait set on a mock community, they found that they detected 24 of 26 genera tested, and that two less abundant species (i.e., *Methanobrevibacter smithii* and *Methanococcus aelocius* at 0.00006%) were missed. In addition, Cariou et al., (2018) tested hybridization capture probes designed by Gasc & Peyret (2018) on a previously characterized pea aphid and found their enriched libraries to be representative of the bacterial population. There are some key differences between the design of our baits set and Gasc & Peyret (2018). Foremost, is the number of baits included in the bait set. Our bait set included 37,745 120mer baits, whereas Gasc & Peyret bait set include 15 baits that are 28- to 50-mer. Using more baits with more sequence variation among the baits helps to capture a greater range of diverse targets and thus generates more accurate abundance estimates of the full range of community members. Having a more extended bait set, as ours, may allow to overcome some of the previous challenges, demonstrated by the ability to detect all genera

in our mock communities. These aspects are critical when studying environmental samples and searching for rare taxa. In addition, the use of longer hybridization times or "double capture" (i.e., when captured product is captured again) can improve the percentage of on target reads and help capture rare sequences. Future work to identify the optimal bait set(s) for various microbial communities and research objectives should include a direct comparison of the Gasc & Peyret (2018) bait set verses our bait set.

Preparing 16S-cap libraries can most readily be accomplished by using an existing enrichment kit, which ranges in cost from $1,500 - $5,200 depending on the number of reactions purchased. To reduce reagent costs and hands-on time, we have successfully pooled multiple samples (see section 2.5), which is commonly done (Glenn and Faircloth, 2016). For example, pooling samples in groups of eight reduces capture costs from $93.75 per sample to $11.72 per sample (Supplementary Table 3). Larger numbers of samples can be pooled to further reduce costs, but there are tradeoffs (see Glenn & Faircloth, 2016). Our baitset is commercially available from Arbor Biosciences in ready-to-use kit format, and the bait sequences are freely available to the scientific community (Supplementary Data 4). Thus, our baits can be modified and/or synthesized by any strategy any researcher desires.

Sequencing 16S-cap libraries require less extensive sequencing than unenriched shotgun metagenomic libraries, which reduces costs (Supplementary Tables 4, 5). For example, a 100-fold 16S-cap enrichment sequenced on an Illumina MiSeq Nano PE150 provides a cost-savings of approximately $315 compared to an unenriched metagenomic shotgun library requiring 1 million reads (Supplementary Table 4). Indeed, 16S-cap makes it economically and logistically reasonable to routinely screen for 16S segments from enriched shotgun metagenomic libraries on Illumina MiSeqs. 16S-cap decreases costs when using a production scale Illumina sequencer (e.g., Illumina NovaSeq) to less than $0.10 per sample when achieving a 100-fold enrichment (Supplementary Table 5). However, because production scale sequencers produce 400 – 2,500 million read pairs, to achieve low cost for samples needing relatively few reads, each run requires huge numbers of samples or a mixture of some samples needing large numbers of reads (i.e., a mixture of projects; see Glenn et al. 2019a). Due to the limited savings possible on production sequencing costs (Supplementary Table 4), the savings in data transfer, storage, and compute time may be more significant than savings in sequencing costs.

In summary, our data demonstrates that the 16S-cap assay and unenriched shotgun metagenomic libraries produce very similar community profiles. Importantly, our 16S-cap library is produced from

a metagenomic library, which eliminates primer (though not all PCR) biases. Additionally, our 16S-cap assay provides a deeper community profile (i.e., more 16S reads that can be queried to a database) with far fewer reads than the unenriched shotgun metagenomic libraries. In environmental samples, we routinely achieved > 400-fold enrichment. Thus, expensive deep sequencing is unnecessary for 16S-cap libraries because a few thousand reads provide the same number of 16S rRNA sequences as millions of shotgun reads. By trading modest additional library preparation costs for reduced sequencing costs (Supplementary Tables 3-5), 16S cap is economical and opens up the possibility of adding deep taxonomic sampling to studies that are capturing other genes of interests (e.g., antibiotic resistance genes (Guitor et al., 2019; Oladeinde et al., 2019; Thomas et al., 2020). In comparison to amplicon libraries, the 16S-cap assay will be more expensive, however, it provides superior microbial community resolution, increased accuracy of relative abundance, and greater flexiblity in terms of sequencer and kit choice. We believe that our bait set is a valuable tool to efficiently and accurately identify microbial community composition and would be well-suited to be used in combination with other bait sets targeting different genes of interest (e.g., antimicrobial resistance baits).

Figures

Figure 1. Overview of data analysis methods on the three library types (i.e., 16s amplicon, 16s hybridization bait capture, and metagenomic libraries).

Figure 2. Relative abundance of bacterial phyla in mock community controls sequenced and analyzed using different methods. Phyla that are not among the nominal composition of the respective mock communities are plotted as black dots next to z_Others. The black dot in the enriched and unenriched library analzyed with 16S mapping software the assigned phyla was Cyanobacteria. In unenriched libraries analyzed with a marker gene approach, the assigned phyla was Ascomycota. Colored vertical bar in each panel represents the nominal abundance of respective phylum. X-axis is plotted in log-scale to show the low abundance phylum. Row panel strips labels identify the mock communities; column panel strips labels identify library type (i.e., amplicon, enriched 16S-cap, unriched metagenomic library) and analyzing strategy (i.e., denoising, 16Smapping, and marker gene).

Figure 3. Relative abundance of bacterial genera in mock community controls sequenced and analyzed using different methods. Panel A is the BEI mock community. Panel B is the Zymo mock community. Genera that are not among the nominal composition of the respective mock communities

were plotted as black dots under z_Others. Colored vertical bar in each panel represents the nominal abundance of respective genus. X-axis plotted in log-scale to show the low abundance genus. Row panel strips labels identify the mock communities; column panel strips labels identify library type (i.e., amplicon, enriched 16S-cap, unriched metagenomic library) and analyzing strategy (i.e., denoising, 16Smapping, and marker gene).

Figure 4. Fold change (i.e., upper or under) comparing the relative abundances of respective genera in each library to its nominal abundance. Duncan's multiple range test was performed to compare each library type for each mock community. Letters indicate whether significant differences were detected.

Figure 5. PCoA plots were constructed using Bray-Curtis dissimilarity matrix at a family level (panel A) and genus level (panel B). Each project is represented by a colored dot (i.e., orange = BEI mock community, green = mouse samples, blue = rat samples, and purple = Zymo mock community). Each library type, sequencing read length and data analysis method is represented by a different shape (i.e., circle = amplicon library, square = 16S-cap enriched PE150 reads, diamond = unenriched PE150 analyzed with 16S mapping and triangle = unenriched PE150 analyzed with metagenome mapping). Numbers represent sample number.

Figure 6. A comparison of the Bray-Curtis distance metric was performed for each library type at a genus level using box plots. Bray-Curtis distance is indicated on the y-axis. Library type is indicated on the x-axis. Duncan's multiple range test was performed to compare each library type for each mock community. Letters indicate whether significant differences were detected.

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Tables

Table 1. A brief overview of the average summary statistics (i.e., number of samples, total raw read-pairs, average filtered/bar, average mapped/filtered) for each sample type of each library type (i.e., 16S amplicon libraries, 16S-cap enriched, and unenriched).

Library Type	Read Length	Sample Type	N Samples	Total Raw Read-Pairs	Total Filtered Reads	Average Filtered/Raw (Mean±SD)	Average Mapped/Filtered (Mean±SD)
Amplicon-16S/V3V4	PE300	Rat feces	5	318,561	247,781	(77.3±6.2)%	NA
Amplicon-16S/V3V4	PE300	BEI Mock	1	136,184	52,734	38.7%	NA
Amplicon-16S/V3V4	PE300	Zymo Mock	1	49,828	24,301	48.8%	NA
Amplicon-16S/V4	PE250	Mice feces	8	526,754	389,000	(77.6±7.1)%	NA
Enriched	PE150	Mice feces	8	8,321,081	11,474,476	(70.1±5.4)%	(59.1±0.8)%
Enriched	PE150	Rat feces	5	6,450,541	9,470,428	(72.9±2.1)%	(57.8±4.1)%
Enriched	PE150	BEI Mock	1	5,345,638	8,203,396	76.7%	70.4%
Enriched	PE150	Zymo Mock	1	3,359,376	5,140,030	76.5%	70.1%
Enriched	PE300	Mice feces	8	1,050,608	1,573,122	(75.1±3.2)%	(59.9±2.1)%
Enriched	PE300	BEI Mock	1	737,309	1,108,481	75.2%	75.7%
Enriched	PE300	Zymo Mock	1	467,250	721,740	77.2%	73.8%
Unenriched	PE150	Mice feces	8	28,219,552	37,894,050	(68.6±6.4)%	0.1%
Unenriched	PE150	Rat feces	5	16,266,683	28,448,468	(87.4±0.9)%	0.1%
Unenriched	PE150	BEI Mock	1	6,263,379	8,889,636	71%	0.2%
Unenriched	PE150	Zymo Mock	1	4,985,957	7,001,503	70.2%	0.2%

Table 2. Summary statistics for simulated data and real data from mock communities, libraries were enriched for 16S using the 16S-cap enrichment and sequenced on an Illumina MiSeq PE150 reads.

Sample ID	Library Type	Avg. No. of (Simulated) Reads	No. of Simulated Reads	Matched Pairs	Matched Forward	Matched Reverse	Total Mapped	Percent of Avg. Total Mapped
Simulated Data								
Zymo Mock	Enriched-PE150	412,520	206,260	171708	190,964	186,216	377,180	91.43%
BEI Mock	Enriched-PE150	415,472	207736	176547	193998	189,777	383,775	92.37%
Lindgreen et al., 2016	Enriched-PE150	490,238	245119	188620	218911	213,918	432,829	88.29%
Real Data								
Zymo Mock	Enriched-PE150	3,904,480	1,952,240	1,314,654	1,548,323	1,503,225	3,051,548	78.15%
BEI Mock	Enriched-PE150	6,260,110	3,130,055	2,127,656	2,486,274	2,435,425	4,921,699	78.62%

Conflict of Interest

The EHS DNA lab provides oligonucleotide aliquots and library preparation services at cost, including some oligonucleotides and services used in this manuscript (baddna.uga.edu). Brian Brunelle and Alison Devault are employed by, and thereby have financial interest in, Daicel Arbor Biosciences, who provided the in-solution capture reagents used in this work.

Author Contributions

TG conceived of the project. JW, JT, TK, BG, KL, and TG designed experiments. JW, TK, and BG performed the experiments. AD and BB designed the baits. JW and NB analyzed the data. AD, BB, KL, OR, JSW, and TG provided funding and resources. MB wrote the manuscript. JW, TK, NB wrote sections of the manuscript. MB and JW produced figures and tables. All authors critically reviewed, edited, and approve of this work.

Funding

Funding for this grant was provided by the Center for Disease Control contract 200-2018-02889 (75D30118C02889), US Department of Energy Cooperative Agreement number DE-FC09-07SR22506, National Institute of Health (R01ES024950, P30ES010126, and P42ES031007) and United States Agency for International Development via Peanut and Mycotoxin Innovation Laboratory (ECG-A00-13-00001-00). Daicel Arbor Biosciences provided the customized in-solution capture reagents used in this work.

Acknowledgments

We acknowledge the contributions of Marissa Howard, Amanda Sullivan, Allison Perry, and Laura Rose.

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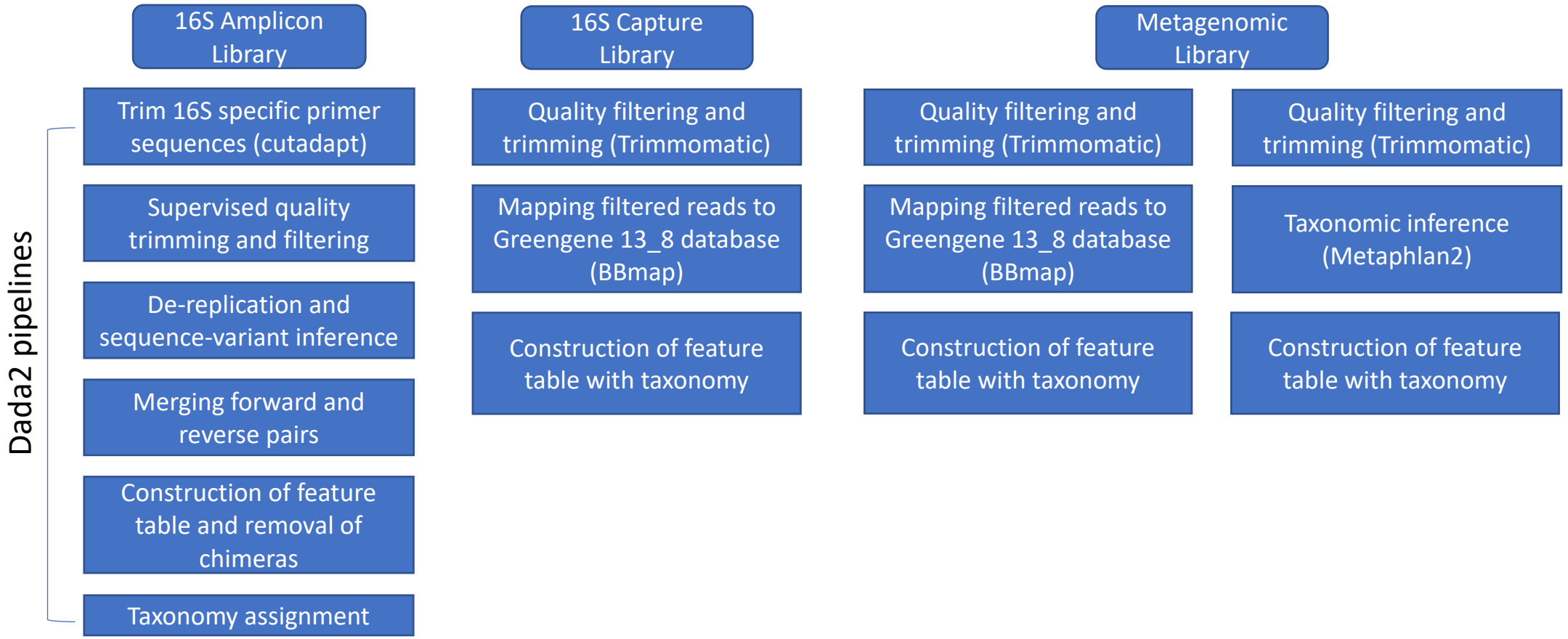
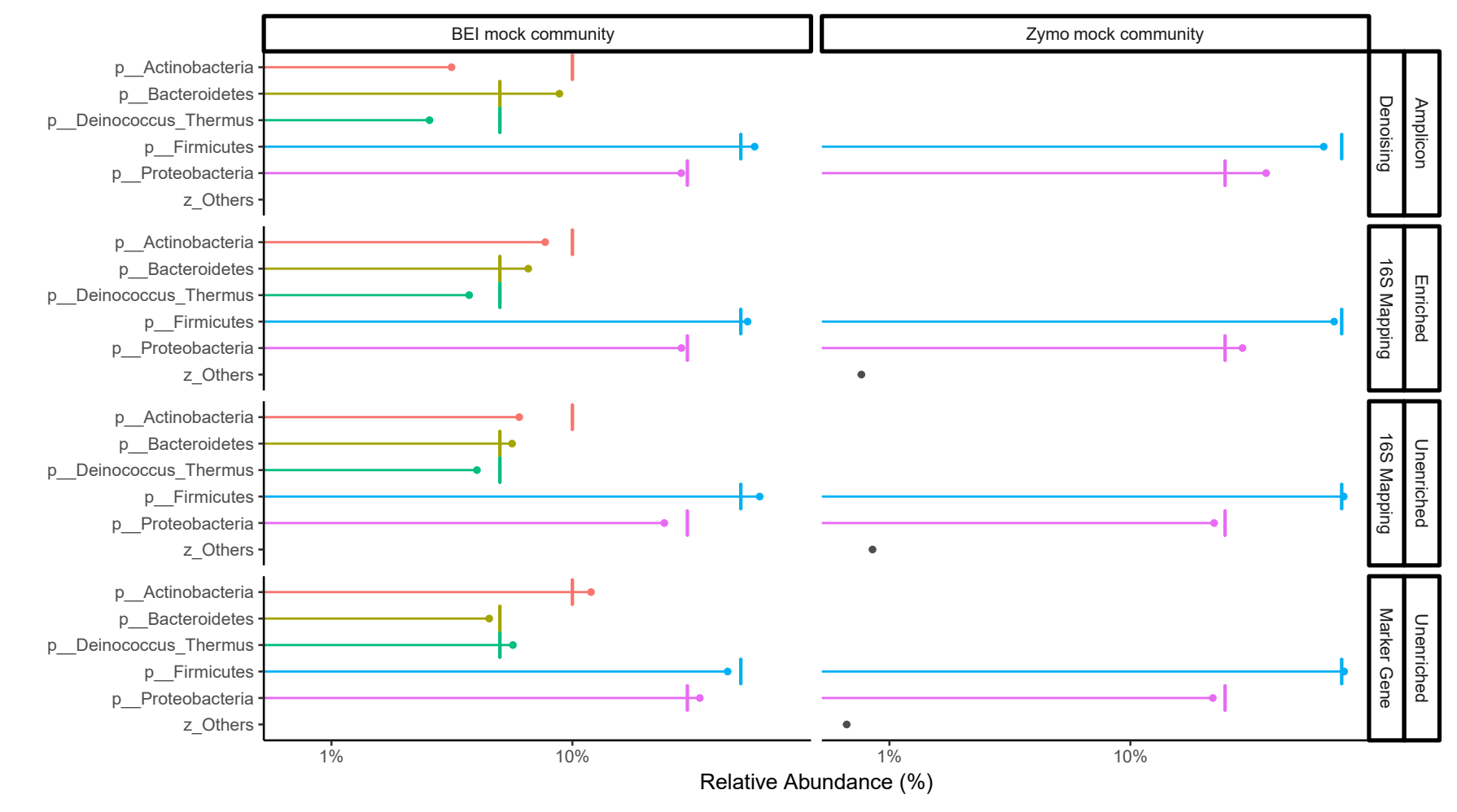
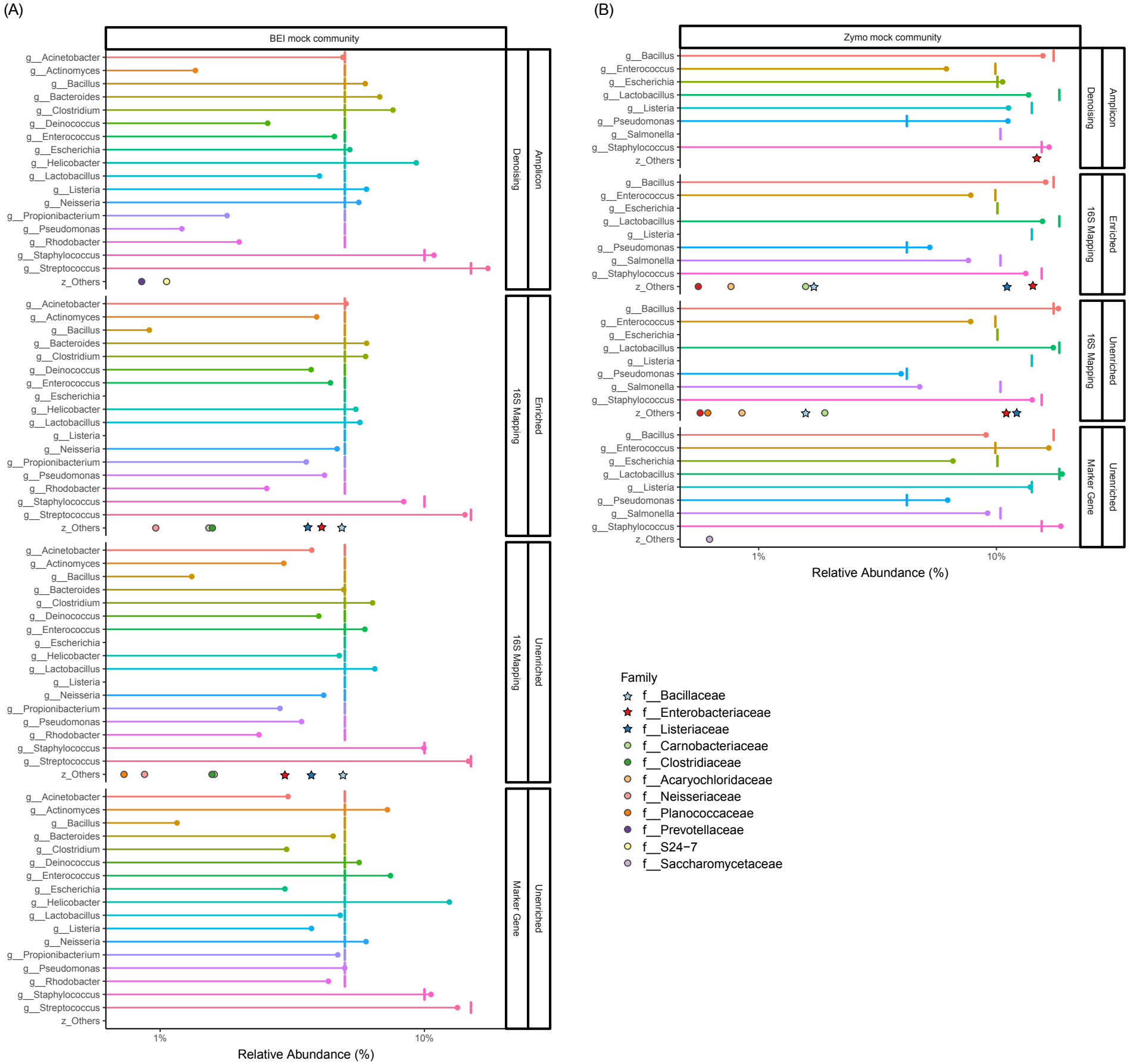
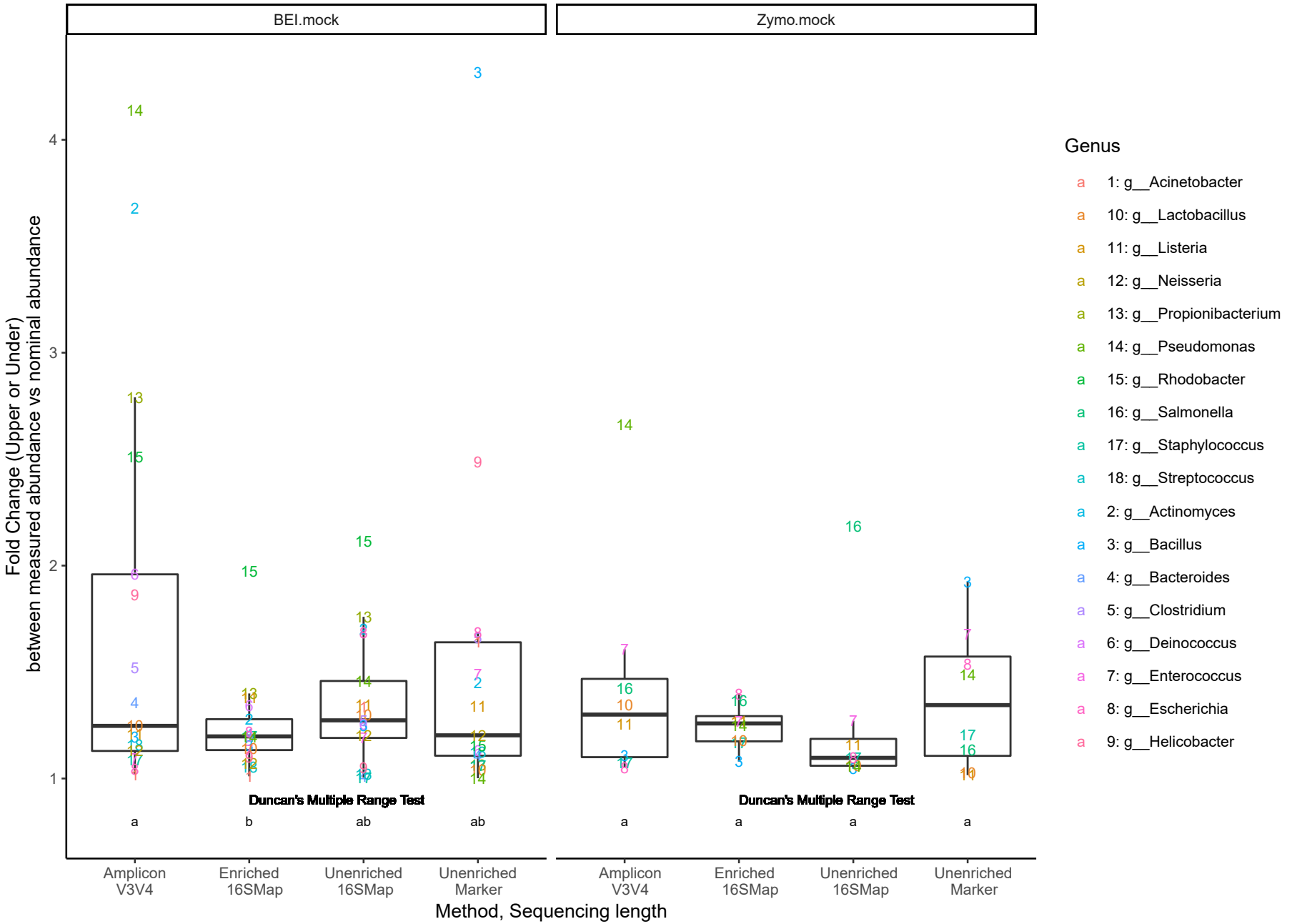
Data Availability

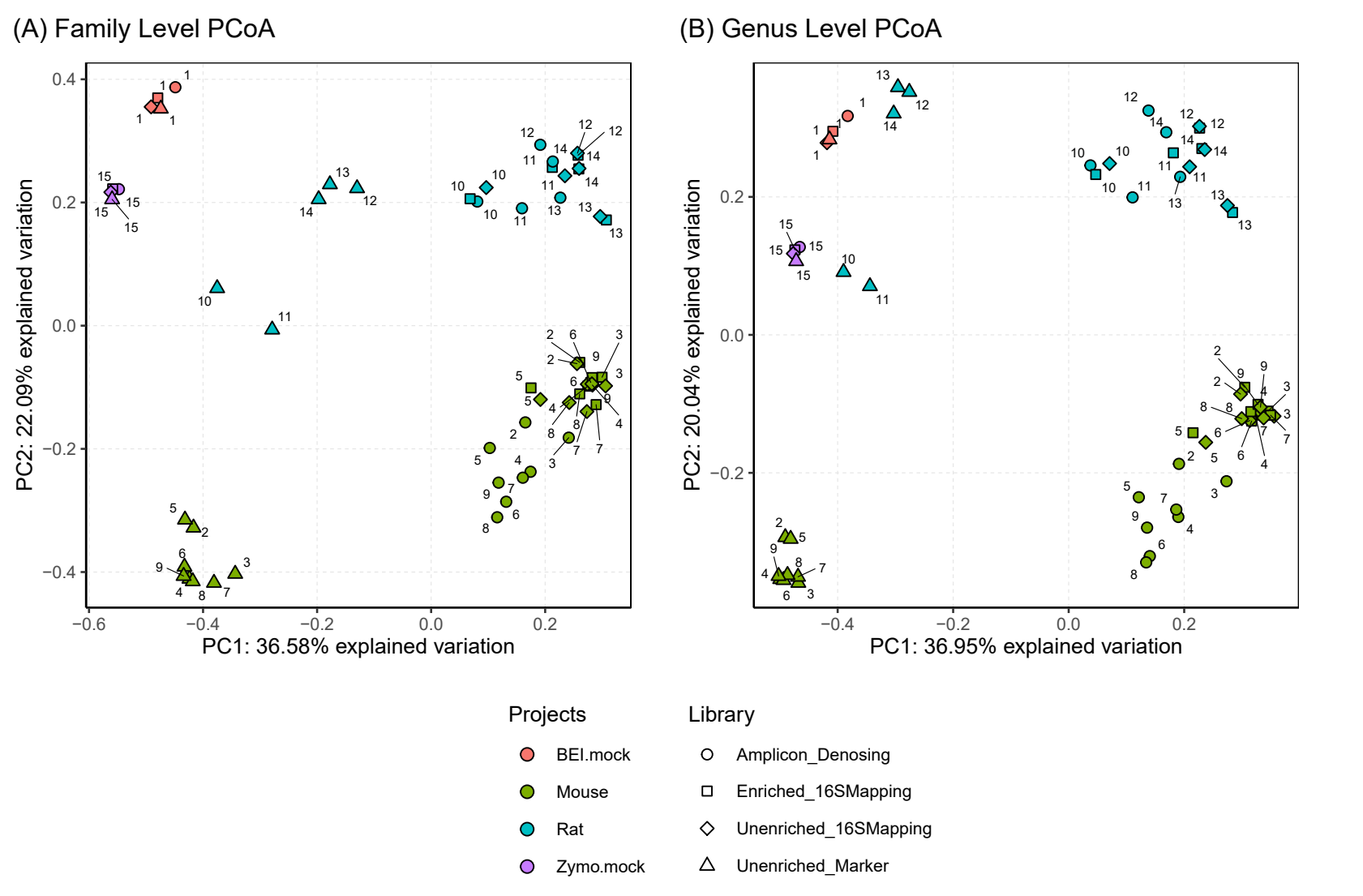
The datasets generated in this study can be found in the authors dropbox https://www.dropbox.com/sh/exg0kow6pyghlmx/AAAIn7R93EawGUDO7TQ6NDIYa?dl=0 .

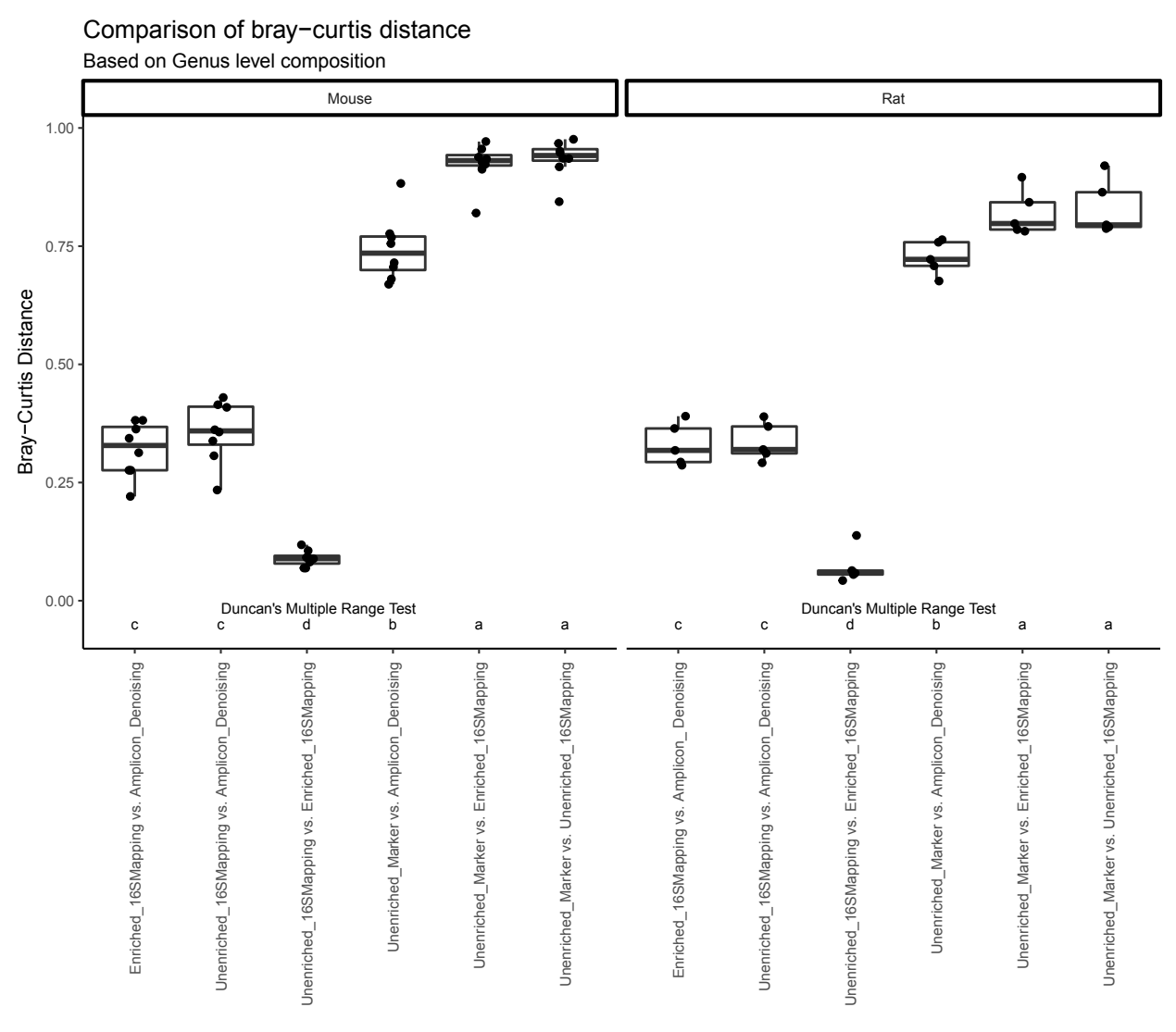

16S Amplicon Library
16S Capture Library
Metagenomic Library
Dada2 pipelines
Trim 16S specific primer sequences (cutadapt)
Supervised quality trimming and filtering
De-replication and sequence-variant inference
Merging forward and reverse pairs
Construction of feature table and removal of chimeras
Taxonomy assignment
Quality filtering and trimming (Trimmomatic)
Mapping filtered reads to Greengene 13_8 database (BBmap)
Construction of feature table with taxonomy
Quality filtering and trimming (Trimmomatic)
Mapping filtered reads to Greengene 13_8 database (BBmap)
Construction of feature table with taxonomy
Quality filtering and trimming (Trimmomatic)
Taxonomic inference (Metaphlan2)
Construction of feature table with taxonomy


BEI mock community
Zymo mock community
Amplicon
Denoising
Enriched
16S Mapping
Unenriched
16S Mapping
Unenriched
Marker Gene
p__Actinobacteria
p__Bacteroidetes
p__Deinococcus_Thermus
p__Firmicutes
p__Proteobacteria
z_Others
1%
10%
Relative Abundance (%)


(A)
BEI mock community
g__Acinetobacter
g__Actinomyces
g__Bacillus
g__Bacteroides
g__Clostridium
g__Deinococcus
g__Enterococcus
g__Escherichia
g__Helicobacter
g__Lactobacillus
g__Listeria
g__Neisseria
g__Propionibacterium
g__Pseudomonas
g__Rhodobacter
g__Staphylococcus
g__Streptococcus
z_Others
Denoising
Amplicon
16S Mapping
Enriched
Unenriched
Marker Gene
1%
10%
Relative Abundance (%)
(B)
Zymo mock community
g__Bacillus
g__Enterococcus
g__Escherichia
g__Lactobacillus
g__Listeria
g__Pseudomonas
g__Salmonella
g__Staphylococcus
z_Others
Denoising
Amplicon
16S Mapping
Enriched
Unenriched
Marker Gene
1%
10%
Relative Abundance (%)
Family
f__Bacillaceae
f__Enterobacteriaceae
f__Listeriaceae
f__Carnobacteriaceae
f__Clostridiaceae
f__Acaryochloridaceae
f__Neisseriaceae
f__Planococcaceae
f__Prevotellaceae
f__S24−7
f__Saccharomycetaceae


BEI.mock
Zymo.mock
Fold Change (Upper or Under)
between measured abundance vs nominal abundance
Duncan's Multiple Range Test
a
b
ab
ab
Duncan's Multiple Range Test
a
a
a
a
Amplicon V3V4
Enriched 16SMap
Unenriched 16SMap
Unenriched Marker
Method, Sequencing length
Genus
a 1: g__Acinetobacter
a 10: g__Lactobacillus
a 11: g__Listeria
a 12: g__Neisseria
a 13: g__Propionibacterium
a 14: g__Pseudomonas
a 15: g__Rhodobacter
a 16: g__Salmonella
a 17: g__Staphylococcus
a 18: g__Streptococcus
a 2: g__Actinomyces
a 3: g__Bacillus
a 4: g__Bacteroides
a 5: g__Clostridium
a 6: g__Deinococcus
a 7: g__Enterococcus
a 8: g__Escherichia
a 9: g__Helicobacter


(A) Family Level PCoA
(B) Genus Level PCoA
PC2: 22.09% explained variation
PC1: 36.58% explained variation
PC2: 20.04% explained variation
PC1: 36.95% explained variation
Projects
BEI.mock
Mouse
Rat
Zymo.mock
Library
Amplicon_Denosing
Enriched_16SMapping
Unenriched_16SMapping
Unenriched_Marker


Comparison of bray−curtis distance
Based on Genus level composition
Mouse
Rat
Bray−Curtis Distance
1.00
0.75
0.50
0.25
0.00
Duncan's Multiple Range Test
c
c
d
b
a
a
Duncan's Multiple Range Test
c
c
d
b
a
a
Enriched_16SMapping vs. Amplicon_Denoising
Unenriched_16SMapping vs. Amplicon_Denoising
Unenriched_16SMapping vs. Enriched_16SMapping
Unenriched_Marker vs. Amplicon_Denoising
Unenriched_Marker vs. Enriched_16SMapping
Unenriched_Marker vs. Unenriched_16SMapping
Enriched_16SMapping vs. Amplicon_Denoising
Unenriched_16SMapping vs. Amplicon_Denoising
Unenriched_16SMapping vs. Enriched_16SMapping
Unenriched_Marker vs. Amplicon_Denoising
Unenriched_Marker vs. Enriched_16SMapping
Unenriched_Marker vs. Unenriched_16SMapping