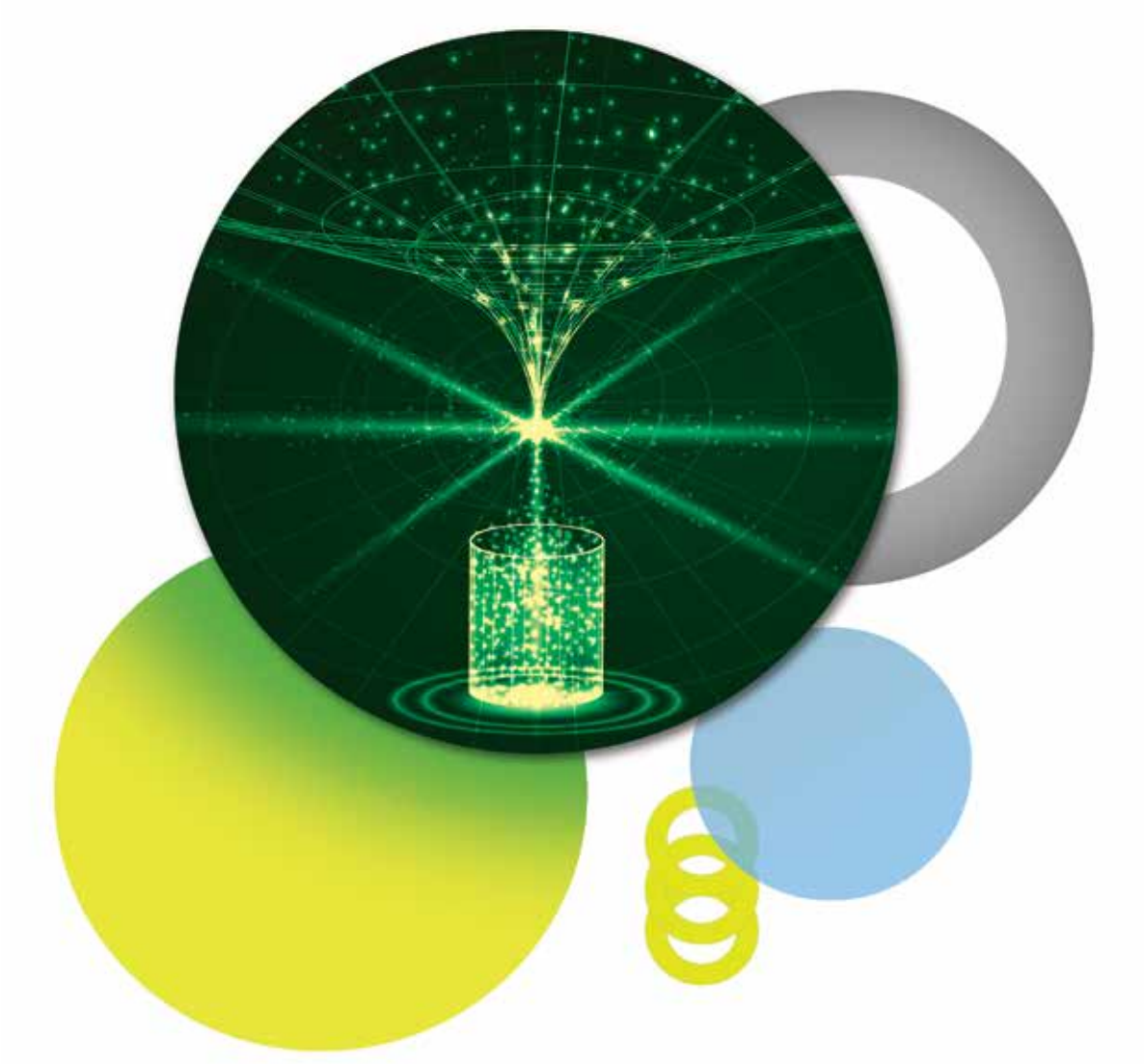
ZYMO RESEARCH

The Beauty of Science is to Make Things Simple®

Development of Human Fecal Microbiome Reference Material and Database



Introduction

The human fecal/gut microbiome is a complex and diverse microbial community that plays an important role in human health. In recent years, there have been innumerable studies that link the gut microbiota to a wide range of conditions such as Alzheimer's Disease, asthma, cancers, and Gestational Diabetes Mellitus (GDM), as well as neurological disorders such as autism, depression, and Parkinson's Disease[1-5]. Microbiome profiling featuring Next Generation Sequencing (NGS) is the essential technology that is underlying these recent advances. Whereas traditional microbiology focuses on one organism at a time, NGS has become a routine analysis for profiling large microbial communities.

However, one persistent problem with the microbial profiling workflow is that seemingly small methodological differences may have considerable impact on the results obtained. This workflow includes sample collection, transportation, storage, DNA/RNA extraction, NGS library preparation, sequencing, and bioinformatic analysis. Due to the lack of strict quality control measures and well-established microbiome standards, researchers have found poor data reproducibility between microbiome datasets generated by different labs. To address these challenges, Zymo Research has released several microbiome standards featuring mock microbial communities with pre-defined compositions. One of which is the ZymoBIOMICS Gut Microbiome Standard (D6331), which contains 21 microbial strains to mimic a gut microbiome. The mock community has an accurate pre-defined composition which makes it ideal for assessing the accuracy and bias of a gut microbiome profiling workflow.

Unfortunately, due to the limited strains that can be included in a mock community, a microbiome standard cannot mimic all aspects of a real fecal sample. An important and undeniable feature of a fecal sample is its high microbial diversity, which represents a significant challenge in both wet-lab and dry-lab parts of a microbiome profiling workflow. To address this limitation, Zymo Research has partnered with the BioCollective to introduce the first whole stool microbiome reference material – The ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology (D6323). 200,000 aliquots have been created that are derived from a huge homogeneous mixture of human feces. Each aliquot contains enough material for 10 uses, allowing for a total of 2 million identical analyses from one sample source. This ensures all sequencing labs have access to the same homogenous human fecal sample.

Characterization

There are several key differences between mock communities such as the ZymoBIOMICS Gut Microbiome Standard (D6331) and microbiome reference materials. Microbiome mock communities are composed of select quantified microbial species, whereas reference materials are native source material, such as feces or soil. The ZymoBIOMICS Fecal Reference with TruMatrix Technology (D6323) consists of real feces with natural diversity. Because it is natural material, and not cultured and quantified in a lab, its true microbial composition is unknown. Thorough sequencing and characterization are required to resolve this complex microbial community and, eventually, with enough depth and consensus, approach a ground truth.

In order to accurately characterize the reference material, an unbiased mechanical lysis process (5 minutes of bead beating with FastPrep-24) was utilized to ensure complete lysis of all microbial cells. DNA and RNA were then extracted and purified, and DNA library preparation was performed using KAPA HyperPlus kit, which we found introduced the least bias among shotgun library prep kits, according to our previous studies. RNA library preparation was performed using Zymo-Seq RiboFree Total RNA Library Kit, and to avoid bias ribosomal RNA depletion was avoided. The DNA and RNA libraries were then sequenced deeply using Illumina sequencing with greater than 30 million reads each. The microbial composition of these two datasets were determined using an in-house bioinformatic pipeline as shown in Figure 1.

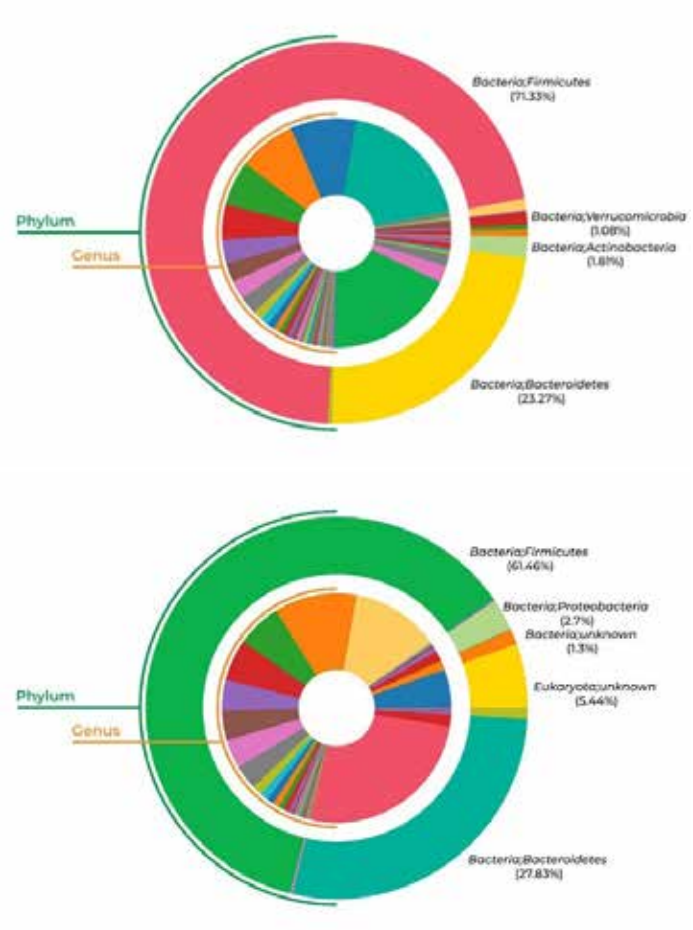


Figure 1 Phylum and genus level taxonomy profiles determined by metagenomic sequencing (top) and metatranscriptomic sequencing (bottom).

Online Portal for Data Sharing

Due to the complexity of this reference material, no single characterization can represent the "ground-truth" composition of the product. The effective profiling of this product requires a joint effort from the whole microbiome research community. Different extraction/purification and library prep methods, sequencing platforms, and bioinformatic tools are required for more exhaustive characterization. Data sharing is necessary and critical to the success of building such a joint effort. To facilitate this, Zymo Research has built an online portal specifically for this fecal reference, to enable sequencing data submission, metadata recording, record searching, and data download. Zymo Research's internal characterization and detailed method description are all in the portal and available to all who sign up. Overtime, as more researchers deposit their characterization data into the portal, this will be an opportunity to approach ground truth and a very valuable public resource for all microbiome researchers. Learn more and access the database at https://www.fecalreferencedb.com/.

Characterization with PacBio HiFi Sequencing

Zymo Research and PacBio have united over a shared goal to advance the field of metagenomics. The collaborative efforts, aim to sequence complex biological samples (e.g. soils and feces), and produce complete genomes for all taxa present in the sample. Zymo Research and PacBio have already achieved over 200 circularized genomes from this fecal reference standard that were assembled using PacBio HiFi sequencing, and additional data is being processed for even greater insight. As both accurate profiling and high molecular weight DNA were considerations, the ZymoBIOMICS DNA Miniprep Kit (D4300) was used for extraction. Lysis was performed with a Vortex Genie II, using 40 minutes of uninterrupted bead beating. The resulting DNA samples have a size of 8-15kb, which can be fed directly into PacBio SMRTbell® library preparation, without additional processing or shearing considerations. Two SMRT cells of PacBio HiFi data were used for the original characterization and additional data generation is still ongoing. The current data is now available in Zymo Research's online public portal.

Assessing Reproducibility and Consistency

The fecal reference material captures the true diversity of a fecal sample, making it ideal for assessing the reproducibility and consistency of a fecal microbiome workflow. It validates microbiome workflows in strictly regulated settings, such as CLIA-CAP and GLP facilities. For example, two studies are provided that assess data reproducibility in a single lab and across multiple labs.

In the first study, DNA extraction was performed from 100 µl of the fecal reference in a single lab. Bead beating was performed on a Vortex Genie II with a horizontal tube adaptor at the maximum speed for 40 minutes and sequenced by 16S rRNA gene sequencing targeting the V3-V4 hypervariable region. This process was repeated six times to collect the data from 6 separate

runs. The microbial composition is shown at the Phylum level and the results show consistent relative abundances across the 6 runs (Figure 2).

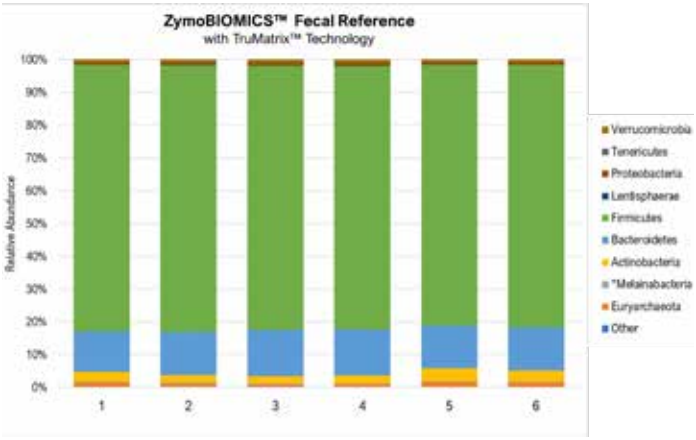


Figure 2: Stability and consistency of taxonomy profiles at the phylum-level of the ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology across different runs of 16S rRNA gene sequencing

In the second study, the fecal reference was used to assess the consistency of microbiome profiling across labs. Four different labs used the same workflow for microbiome profiling except for a small variation in the microbial lysis process: a different mechanical lysis device was used by each lab. The profile generated from Lab Q appears to have more deviation compared to others. It has a lower abundance of Firmicutes, which are Gram-positive and therefore, generally tougher to lyse compared to Bacteroidetes and similar easy-to-lyse Gram-negative bacteria. This is a common indication that the microbial lysis is incomplete, resulting in the overestimation of the abundance of easy-to-lyse microbes, and underestimation of difficult-to-lyse microbes.

The ZymoBIOMICS Fecal Reference with Trumatrix technology is a true diversity human stool reference material in sufficient quantity for all microbiome researchers to validate and assess consistency of sample processing and compare to other researchers results on the same sample material. The Fecal Reference database prepared specifically for this microbiome reference material provides an easy way to access data of other researchers and share your characterization.

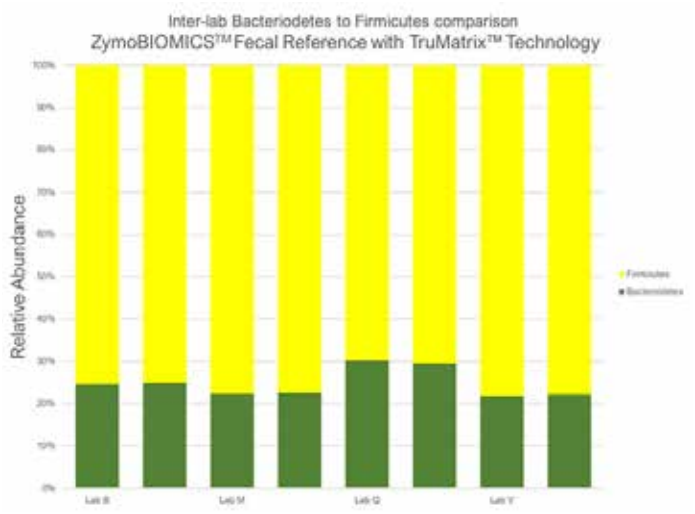


Figure 3: Inter-lab data comparison utilizing different bead beater devices. Phylum-level taxonomic profiles of the ZymoBIOMICS™ Fecal Reference Material with TruMatrix™ Technology were generate with metagenomic sequencing

References:

1. **Halfvarson J, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nature Microbiology. 2017, 2: 17004.**
2. **Rooks MG, et al. Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. ISME Journal. 2017, 8: 1403-1417.**
3. **Bullman S, et al. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. Science. 2017, 358(6369): 1443-1448.**
4. **Sampson TR, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell. 2016, 167(6): 1469-1480.**
5. **Zheng P, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Molecular Psychiatry. 2016, 21: 786-796.**
6. **Costea et al. Towards standards for human fecal sample processing in metagenomic studies. Nat biotechnol 2017;35: 1069-1067**