Title:

Variability and Bias in Microbiome Metagenomic Sequencing: an Interlaboratory Study Comparing Experimental Protocols

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Abstract

Background

Several studies have documented the significant impact of methodological choices in microbiome analyses. The myriad of methodological options available complicate the replication of results and generally limit the comparability of findings between independent studies that use differing techniques and measurement pipelines. Here we describe the Mosaic Standards Challenge (MSC), an international interlaboratory study designed to assess the impact of methodological variables on the results. The MSC did not prescribe methods but rather asked participating labs to analyze 7 shared reference samples (5x human stool samples and 2x mock communities) using their standard laboratory methods. To capture the array of methodological variables, each participating lab completed a metadata reporting sheet that included 100 different questions regarding the details of their protocol. The goal of this study was to survey the methodological landscape for microbiome metagenomic sequencing (MGS) analyses and the impact of methodological decisions on metagenomic sequencing results.

Results

A total of 44 labs participated in the MSC by submitting results (16S or WGS) along with accompanying metadata; thirty 16S rRNA gene amplicon datasets and 14 WGS datasets were collected. The inclusion of two types of reference materials (human stool and mock communities) enabled analysis of both MGS measurement variability between different protocols using the biologically-relevant stool samples, and MGS bias with respect to ground truth values using the DNA mixtures. Owing to the compositional nature of MGS measurements, analyses were conducted on the ratio of Firmicutes: Bacteroidetes allowing us to directly apply common statistical methods. The resulting analysis demonstrated that protocol choices have significant effects, including both bias of the MGS measurement associated with a particular methodological choices, as well as effects on measurement robustness as observed through the spread of results between labs making similar methodological choices. In the analysis of the DNA mock communities, MGS measurement bias was observed even when there was general consensus among the participating laboratories.

Conclusion

This study was the result of a collaborative effort that included academic, commercial, and government labs. In addition to highlighting the impact of different methodological decisions on MGS result comparability, this work also provides insights for consideration in future microbiome measurement study design.

Introduction

Over the last decade, advances in DNA sequencing technology (Next-Generation Sequencing or NGS) have led to its widespread adoption by the scientific community for myriad applications. One such application, known as metagenomic sequencing (MGS), has led to a transformation in how we measure and characterize complex microbial communities of microbiomes. MGS has

emerged as an important and powerful tool as we seek to comprehend the roles of microbes inside complex and dynamic communities that are both capable of maintaining and harming human and environmental health. MGS measurements are able to 'see' whole classes of microorganisms present in a microbiome sample (e.g., all bacteria by 16S rRNA gene amplicon sequencing (16S), or all dsDNA by whole-genome shotgun WGS); MGS can also assign a relative abundance to each microorganism in complex samples. [1-4] Because of these advantages, MGS is being increasingly adopted across diverse application spaces including infectious disease diagnostics, [5-12] epidemiological investigations, [13-15] food safety, [16] and biothreat surveillance. [9, 17-19]. The results of MGS measurements have been used to diagnose infectious diseases that were missed by conventional methods.[20, 21] As such, regulatory agencies are actively developing new guidance and policies regarding the use of MGS in the clinic and in other regulated spaces.

While MGS measurements hold great promise in monitoring and understanding microbial communities, the current impact is often hampered by a lack of reproducibility and comparability, particularly between different research centers. [22-24] MGS measurement results are the product of complex workflows incorporating multiple distinct steps and involving a multitude of methodological choices (e.g., sample collection and storage, DNA extraction and purification, NGS library preparation either for WGS or 16S, DNA sequencing platform, data cleanup and processing, bioinformatic analysis, interpretation). Throughout this workflow, measurement bias (deviation from ground truth) and measurement noise (experimental variability) are potentially introduced with each step and will depend on the particular methodological choices made.[25] It is widely recognized that the interlaboratory reproducibility of MGS microbiome measurements is poor, and there have been numerous efforts aimed at benchmarking the analytical performance of MGS measurements in terms of sensitivity, specificity, precision, reproducibility, etc. [26-33] These challenges are well-documented, and the community has long recognized the need for studies to prioritize and investigate the sources of variability and bias in the experimental workflow [27] and the need for standardized materials and methods to improve the comparability and scope of MGS measurement results.

Designing the studies to identify sources of variability and bias as outlined above comes with its own set of challenges including: sufficient numbers and diversity of reference samples to help power the study; testing of a wide range of variables; a lack of consistent data analysis; cost & coordination. While the task may seem daunting, several groups have taken up the call to begin to address these challenges. In recognition of the complexity of the workflow, some groups have broken the MGS workflow into more manageable sections with most of the focus being directed at characterizing the effect of data processing and analysis either using *in silico* datasets [32, 33] or metagenomic DNA control material [26, 28, 34-38]. Other groups have sought to capture bias throughout the workflow by distributing sets of identical microbiome samples [39, 40].

Herein, we describe the Mosaic Standards Challenge (MSC). The MSC brought together academic, federal, and private industry partners in an international interlaboratory study focused on capturing the diversity of protocols and methodological choices involved in NGS-based microbiome measurements and understanding their impact on observed taxonomic

profiles. To achieve this, we produced a panel of homogeneous microbiome samples, developed a custom cloud-based web portal for collecting sequencing data and metadata, [41, 42] and statistically evaluated the MGS results. The microbiome samples included human feces from multiple donors and DNA mock communities. For every sample analyzed, nearly 100 metadata parameters describing the MGS protocol were collected, with participation from 44 MGS laboratories. The resulting analysis demonstrated that various protocol choices have significant effects that range from skewing MGS measurement results (e.g., WGS or 16S analysis) to increasing measurement robustness (e.g., homogenizer use during DNA extraction). The ground truth DNA mock community samples revealed that MGS measurement bias can persist, even when there is consensus (measurement agreement) among results from different laboratories.

Results

Briefly, the study consisted of three components: reference material selection and production, broad participation from the microbiome community including metadata reporting and MGS data uploads, and common analysis pipelines applied to the raw sequencing data alongside the methodological metadata from each participating laboratory. The timeline and overall workflow of the MSC are shown in Figure 1.

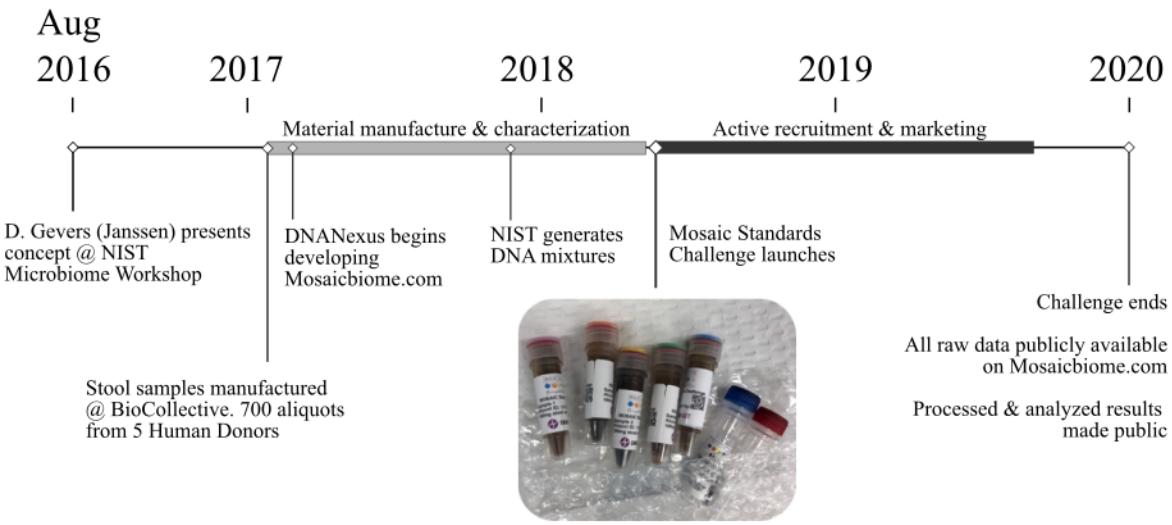

Figure 1 Study design timeline. Inset image shows material received by participants.

Material Production

The reference samples selected and distributed in this study included 5 human stool samples and 2 DNA mixtures (mock DNA communities). The five stool samples were selected from a pool of potential donors based on the dissimilarity of their microbiome composition (Figure 2).

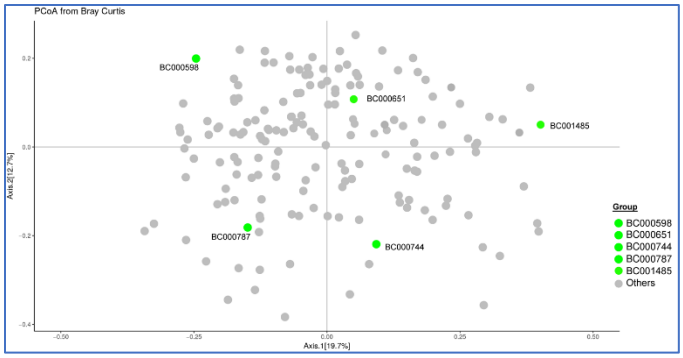

Figure 2 Principal coordinate analysis of donor samples in the BioCollective stool collection. Stool samples included in the Mosaic study (green points) were selected based on their PCoA diversity within the constellation of samples available from the BioCollective. All selected donors self-reported being healthy except BC001485, who reported Parkinson's Disease.

For each sample, multiple stool donations from an anonymous individual were homogenized in the presence of a stabilization buffer to produce 1-liter of homogenized, stabilized fecal material. Two allochthonous microorganisms, *Aliivibrio fischeri* and *Leifsonia xyli,* were also added to each batch of stool (~10^8 cells/mL) and homogenized. Approximately 700 aliquots (1 ml per aliquot) were prepared from each of the 5 batches, and aliquots were stored at -20 °C until ready to ship to participants. To verify that these materials were sufficiently homogenous, 10 aliquots were selected randomly from each of the 5 batches and subjected to both 16S and WGS analysis. These results (Figures 3 and Si-1) indicate that (i) each individual sample donor has a unique microbiome composition, and (ii) the stool samples are suitably homogenized (fit-for-purpose).

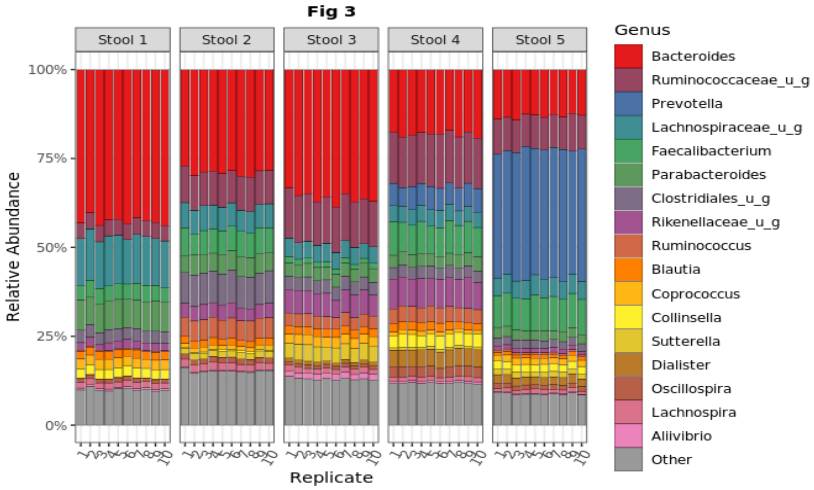

Figure 3 Metagenomic sequencing analysis of Mosaic stool samples to determine homogeneity of samples. The bar chart shows the relative abundance as measured by 16S rRNA MGS at the genus level for 10 replicate tubes from each stool sample (stool 1-5). Taxa colors denote the 17 most abundant genera overall, as well as an exogenously added internal standard; all other genera are grouped as 'other' and shown in grey. MGS analysis by WGS also exhibited good homogeneity (Figure SI-1).

Additionally, 2 DNA-based mixtures were prepared for the MSC where ground-truth taxa abundances could be assigned. Both materials (Mix A and Mix B) were mixtures of genomic DNA (mock communities) that were extracted from pure cultures of 13 bacterial species mixed at roughly equal genomic ratios (Mix A) or at varying abundances across 3-orders of magnitude (Mix B).

174 **Recruitment and Community Participation**
175 To kick-off the study, a targeted media campaign was launched to recruit participation in the
176 MSC; study enrollment was open from May 2018 until December 2019. [43, 44] Each lab that
177 volunteered to participate was shipped the 5 stool samples and two DNA samples free of
178 charge. By design, the MSC did not prescribe any required methods or instrumentation to the
179 participants. Rather, participants were instructed to use their own in-house protocols and
180 encouraged to explore new methods. To capture these methodological details, a
181 comprehensive standardized metadata reporting sheet was developed and deployed where
182 participants could record the details of their protocols. This metadata reporting form included
183 over 100 questions and was intended to capture the most intricate details of each step in the
184 measurement process (The metadata capture questions are available in the supplemental file
185 1.) Both methodological data and raw data were then captured using a custom web-based
186 cloud analytics portal that enabled the collection, storage, analysis, and visualization of MGS
187 data generated by the MSC participants [41]. This not only facilitated analysis within a single
188 bioinformatics pipeline; it also enabled participants to view their results in the context of all
189 other MSC results immediately following upload. Thus, participants could quickly visualize how
190 their methods compared to others in the community.

191 A total of 44 labs participated in the MSC by submitting MGS results (16S or WGS) along with
192 accompanying metadata (Table 1). Most labs analyzed all samples, though some only analyzed
193 the stool samples. Of the 44 MGS submissions, 30 were 16S rRNA datasets and 14 were WGS
194 datasets (Table 1). On average, 16S rRNA MGS datasets had $\geq 10^5$ reads, while the WGS analyses
195 were typically a log higher with $>10^6$ reads (Figure SI-2). Significant variation in read number
196 was observed both between participating labs and individual samples (Figure SI-2).

197 *Table 1 A total of 44 labs submitted 16S or WGS analyses of the Mosaic Stool and DNA samples with accompanying metadata.*
198 *Two sequencing datasets with incomplete metadata were dropped.*

MGS Analysis	Samples analyzed	Number of Labs
16S	5x Stool	8
	2x DNA	0
	7x (Stool and DNA)	22
WGS	5x Stool	0
	2x DNA	0
	7x (Stool and DNA)	14

199
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203 **Metagenomic Sequencing (MGS) Interlaboratory Comparison**

204 A Bray-Curtis principal coordinate analysis (PCoA) for both the 16S (n=150) and WGS (n=70)
205 datasets (Figure 4) demonstrate that the biological variability (i.e., stool sample ID) was the
206 major factor influencing the overall ordination of the data, as expected. The impact of
207 methodological variability can be seen via the dispersal of datasets within each stool sample.

From the PCoA plot of the 16S data (Figure 4a), we observed that one of the participating labs made an apparent transposition in the labeling of samples 3, 4, and 5. Based on this apparent error, we excluded all the data from this lab for the remainder of the analyses described in this manuscript.

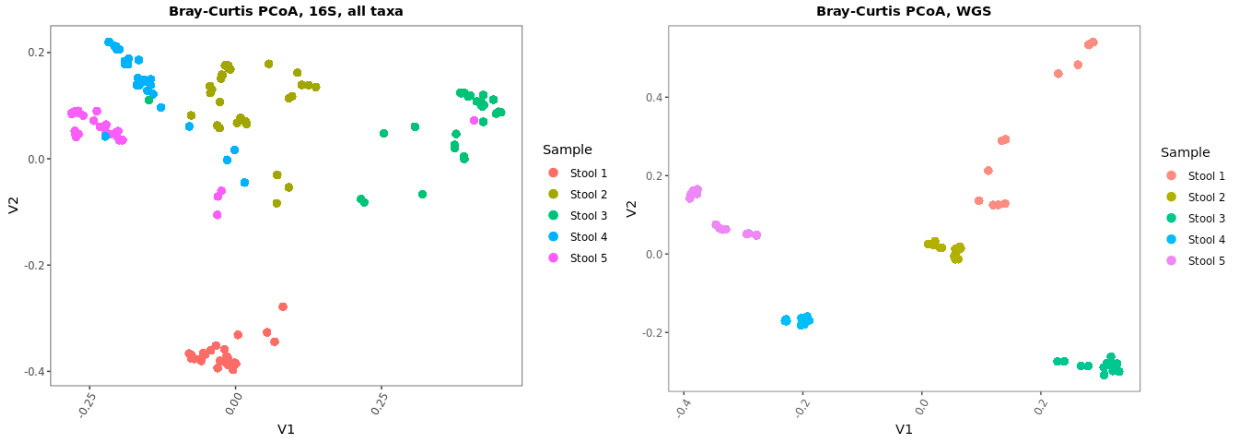


Figure 4: Principal coordinate plots of the Bray-Curtis dissimilarities for 16S and WGS analyses exhibits clustering by Stool sample. Each data point represents a distinct laboratory analysis of each sample. The separation in the clusters is attributed to methodological differences between labs.

<u>Firmicutes:Bacteroidetes ratio</u>

Because of the compositional nature of MGS results, individual taxa relative abundances are not directly comparable between different samples.[45, 46] Instead, ratios of taxa within each sample were expected to be more reliable because the effects of sample composition on each taxa relative abundance could cancel out. One ratio that has been of interest in the field is the ratio of phyla Firmicutes:Bacteroidetes; therefore, we chose this ratio to demonstrate the utility of using ratios of taxa to compare data between samples [39-42]. Thus, this ratio was utilized and included in our results purely for its bioinformatic utility and is not intended to serve as an indicator of gut health or dysbiosis. The Firmicutes:Bacteroidetes ratio was calculated for each Mosaic stool sample and compared among the individual laboratory results (Figure 5). As was expected since each laboratory used their individual MGS protocols (e.g., methodological choices for DNA extraction, library preparation, and sequencing), the Firmicutes:Bacteroidetes ratio varied substantially both between stool samples within each lab, as well as between labs.

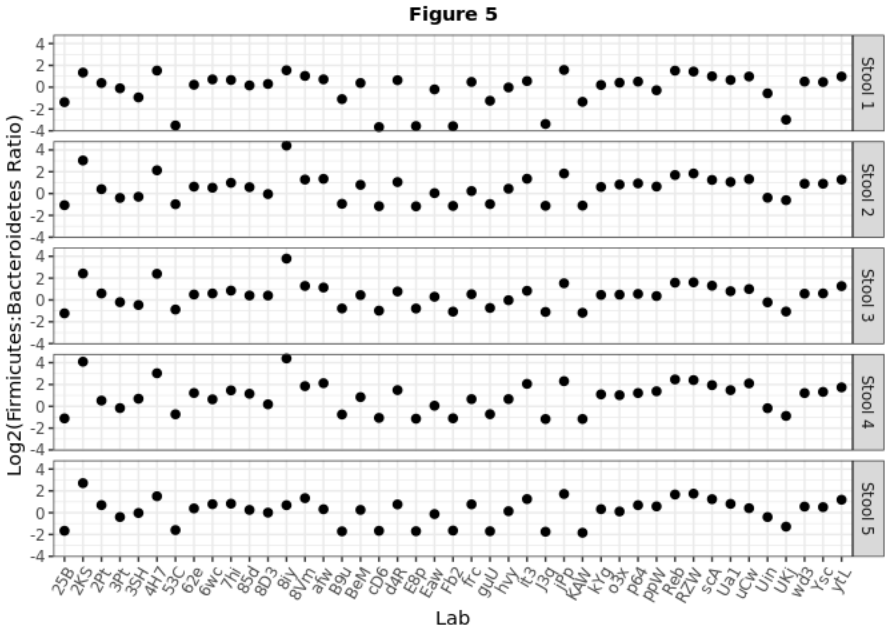


Figure 5. The Firmicutes:Bacteroidetes ratio was calculated for all stool samples and plotted for each participating laboratory. Of note, data submission was anonymous, so multiple submissions from the same research center would appear as distinct labs.

<u>Amplicon vs. Shotgun sequencing</u>

One goal of the MSC was to determine how the selection of different methodological parameters during MGS would lead to observed differences in the taxonomic profiles and relative abundances. The highest-level methodological choice was between 16S MGS or WGS MGS. Indeed, the Firmicutes:Bacteroidetes ratio was affected by the type of sequencing performed, with 16S MGS analyses reporting significantly higher Firmicutes:Bacteroidetes ratios (Figure 6a). While the majority of the 16S MGS datasets indicated that Firmicutes were present at a higher relative abundance than Bacteroidetes, WGS data found the inverse with Bacteroidetes being present at a higher relative abundance than Firmicutes. The magnitude of this effect was quantified by averaging the results from all labs reporting each methodological parameter (e.g., 16S or WGS for sequencing strategy) divided by the average result overall and plotted as a fold change on a log scale (Figure 6b). The dependence of the Firmicutes:Bacteroidetes ratio on analysis strategy that was observed in this dataset could explain recent reports that question the reliability of the Firmicutes:Bacteroidetes ratio as a diagnostic indicator of gut health [47]. This dependence was consistent across all five stool samples (Figure SI-3), and an analysis of each sample's alpha diversity also yielded a similar stratification with respect to the methodological choice of 16S or WGS analysis (Figure SI-4).

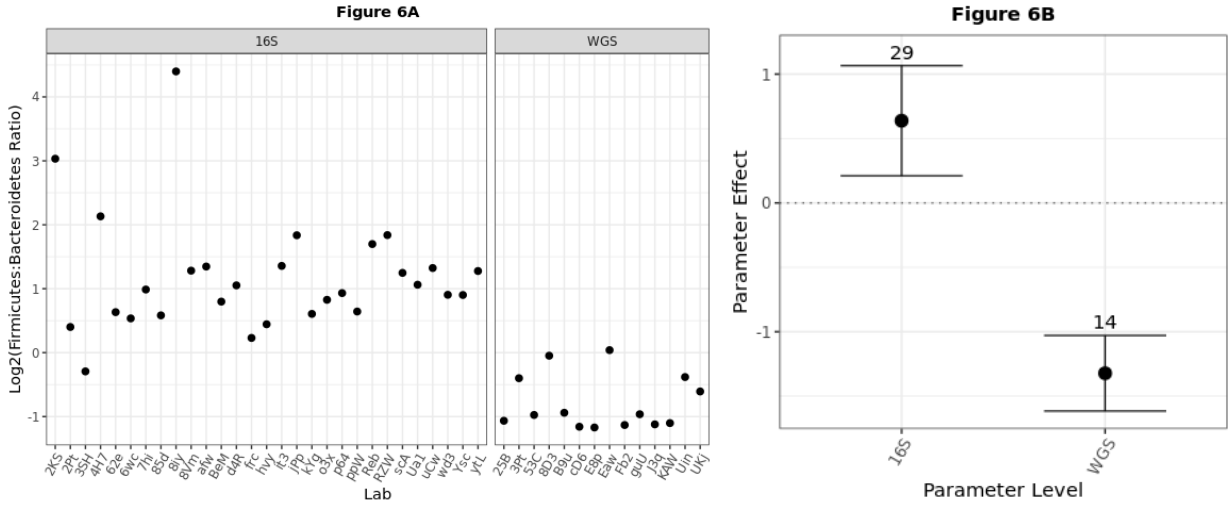
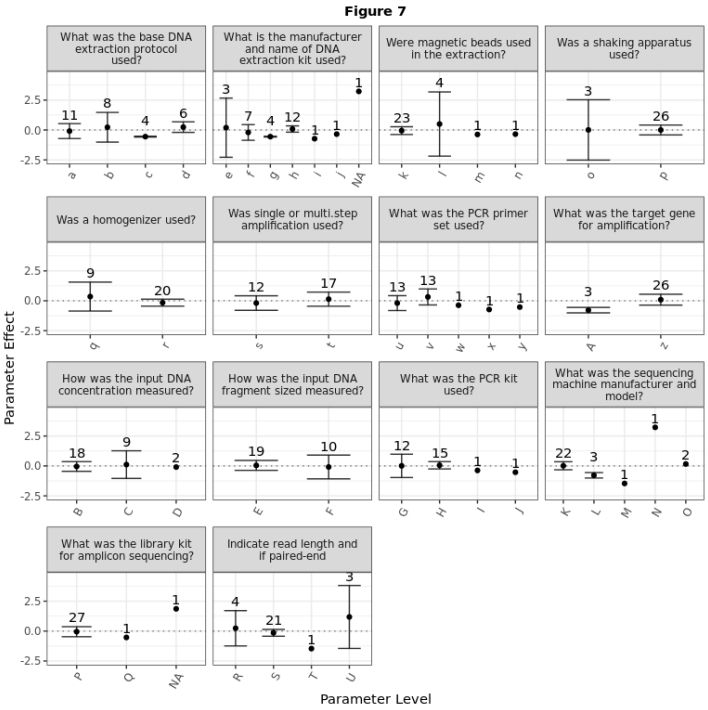


Figure 6 The effect of analysis strategy (16S versus WGS) on the Firmicutes:Bacteroidetes ratio was readily observed for just one stool sample by simple grouping (A), and the effect was quantified (B) by dividing the average results among labs reporting the specified parameter level by the average results overall. In B, this parameter effect was plotted on a log (base 2) scale, such that the horizontal line at 0 denotes the null hypothesis of no effect; error bars show the 99% confidence interval. Quantified effects for the other stool samples were similar and are included in Figure SI-3. Similar stratification was observed when measuring each sample's Inverse Simpson alpha diversity (Figure SI-4).

<u>Other metadata parameters</u>

When submitting results, participating labs were asked to complete a standardized metadata reporting sheet that included 100 different questions regarding the details of their protocol. Some questions were generally applicable like "what sequencing instrument did you use" while others were more nuanced like "what was the PCR primer set used." As such, some fields were required, and others were optional. Because of the large impact generated by the 16S vs. WGS methodological variable (Figure 6) and the hierarchical nature of other methodological choices (e.g., 'What was the target gene amplicon'), we chose to analyze each data set separately. The effect on the Firmicutes:Bacteroidetes ratio on the 16S MGS results was quantified for each subsequent methodological choice (Figure 7) in a similar manner to that employed in Figure 6b. While there were many methodological variables that appeared to have a significant impact on the results (Figure 7; similar analysis for other stool samples is included in Figure SI-5), many of these were only reported by a single lab (n=1). Of the 30 labs submitting 16S MGS data, there were 14 methodological differences in their protocols. Of the 14 labs submitting WGS data, there were 9 methodological differences in their protocols (Figure SI-6). Not all methodologic variables had a significant impact on the result. Methodological variables that were observed to have a significant impact on the 16S MGS results for 2 or more stool samples (parameter effect and 99% confidence interval) included the manufacturer of the DNA extraction kit and the target gene for amplification (Figure 7 and SI-5). Methodological variables that were observed to have a significant impact on the WGS results (parameter effect) for 2 or more stool samples included the DNA extraction protocol, the manufacturer of the DNA extraction kit, and the library kit for shotgun sequencing. In addition to their impact on the parameter effect as described above, some methodological variables were observed to have a significant impact on the robustness of the measurement (observed as a lack of variability when other parameters are varied). For example, when asked whether a homogenizer or shaking

apparatus was used, those labs that reported "Yes" displayed much greater robustness than those reporting "No" (Figure 7 and SI-5 and SI-6).



Symbols	Parameter	Option
a, b, c, d	What was the base DNA extraction protocol used?	(a) Internal, (b) Others, (c) HMP, (d) IHMS
e, f, g, h, i, j	What is the manufacturer and name of DNA extraction kit used?	(e) In-house protocol, (f) No-kit Other method, (g) Mo Bio DNeasy PowerSoil Kit, (h) QIAGEN QiAmpStoolMinikit, (i) Chemagic DNA Blood Kit, (j) Promega Maxwell
k, l, m, n	Were magnetic beads used in the extraction?	(k) glass_beads_0,1mm, (l) No, (m) silica_beads_0,1mm, (n) ceramic_beads_>1mm
o, p	Was a shaking apparatus used?	(p) No, (q) Yes
q, r	Was a homogenizer used?	(q) No, (r) Yes
s, t	Was single or multi.step amplification used?	(s) Single, (t) Multi
u, v, w, x, y	What was the PCR primer set used?	(u) NA, (v) V4 515F/ 806R, (w) Schloss 2013, (x) 318F/806R, (y) EMP V4 515F/806R
z, A	What was the target gene for amplification?	(z) 16S rRNA, (A) Other
B, C, D	How was the input DNA concentration measured?	(B) PicoGreen, (C) Qubit, (D) Other
E, F	How was the input DNA fragment sized measured?	(E) Agilent Bioanalyzer, (F) Other
G, H, I, J	What was the PCR kit used?	(G) Other, (H) Kapa HI Fi Polymerase, (I) NEB Q5 Polymerase, (J) AccuPrime Taq Polymerase High Fidelity
K, L, M, N, O	What was the sequencing machine manufacturer and model?	(K) Illumina MiSeq, (L) Illumina NextSeq 500, (M) Thermofisher Ion Torrent S5, (N) Other, (O) Illumina MiniSeq
P, Q	What was the library kit for amplicon sequencing?	(P) Other, (Q) QIAseq 1-Step Amplicon Library Kit
R, S, T, U	Indicate read length and if paired-end	(R) 100 PE, (S) 150 PE, (T) 100 SE, (U) 250 PE

Figure 7 Within labs performing 16S amplicon sequencing, the parameter effect on the Firmicutes:Bacteroidetes ratio was calculated as described in Figure 6 for each relevant metadata parameter. Shown here from just one stool sample, results from other stool samples were similar and are provided in Figure SI-5.

'Spike-in' organisms

An additional attribute of the fecal materials used for this interlaboratory study was the inclusion of two exogenous organisms to serve as whole-cell internal controls (i.e., spike-ins). Since these organisms were added during the bulk homogenization step, their abundance should be constant across all the stool sample aliquots. As such, it was expected that the ratio of *A. fisherii* to *L. xyli* would be constant for each particular methodology (e.g., within a lab). Surprisingly, *L. xyli* was not identified in any of the submitted 16S datasets and was only observed at a low abundance (approximately 0.001 %) by WGS analysis. When the *A. fisherii*:*L. xyli* ratio (by WGS) was plotted for each participating laboratory (Figure SI-7), significant variability between samples was observed. These data were unexpected and could have resulted from poor database representation of *L. xyli* in the commercially available bioinformatic pipeline used, inefficient DNA extraction, or low or inconsistent distribution during material manufacture, among other possible explanations.

Genomic DNA mixtures

Another control included in the interlaboratory study were mixtures of purified microbial genomic DNA. These were included alongside the stool samples in the Mosaic Kit to serve as parallel processing controls and included two different mixtures, one equigenomic between taxa (Mix A) and one with ten-fold dilutions of the various taxa (Mix B). These genomic DNA mixtures were validated for genome copy number using ddPCR (droplet digital PCR) and serve as 'ground truth' for the MGS measurements. For comparison to the MGS measurements,

genome copy number (as measured by ddPCR) was scaled by the assembled genomes of the individual strains (i.e., rRNA copy number or genome size) to yield ground truth values for comparison to 16S or WGS results, respectively. As with the Firmicutes:Bacteroidetes ratio described above, ratios of individual taxa were used to characterize the DNA mock communities and remove the compositional dependence of the raw relative abundance assignments. Since these analyses included 16S sequencing results, we focused on strains that were unique at the genus level, yielding 6 distinct ratios within each sample. The independent determination of actual DNA concentration (ddPCR) was compared to the results of MGS analyses (Figure 8). While there was some agreement among participating laboratories (consensus), their results generally differed from the actual abundances. Overall, this indicates that even when consensus exists among MGS results, significant unidentified bias can remain. Further, this was taxa-dependent, with some taxa (e.g., Kp by 16S or Pa by WGS) producing particularly significant variability and deviation from ground truth.

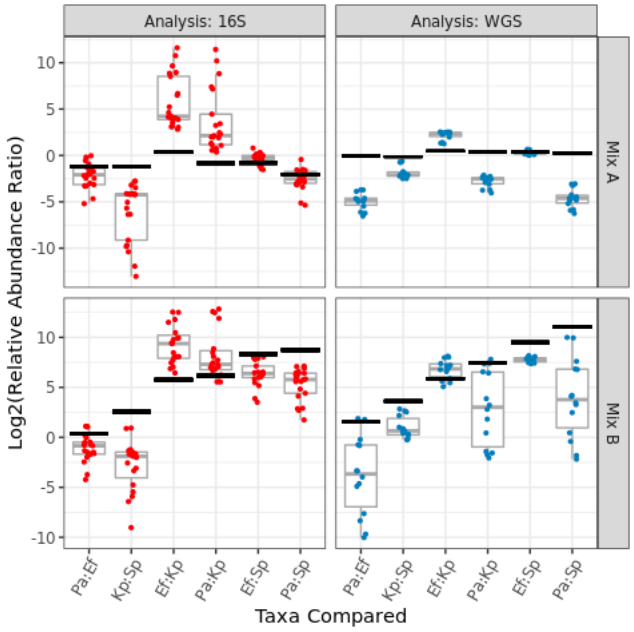


Figure 8 For the DNA mixtures, independently measured 'ground truth' results (black 99% confidence intervals) for the ratios between taxa relative abundances can be compared to each individual lab's amplicon (red points) or shotgun (blue points) metagenomic sequencing results, as well as the range of results (grey boxplots) among participating labs. The taxa in Mix A were roughly equally abundant, while the Mix B sample exhibited groups of taxa added at 10-fold dilutions. The horizontal axis identifies the taxa (known to be present in the DNA mixtures) whose observed relative abundances were ratioed. The ground truth values were scaled to account for known 16S copy numbers (for amplicon sequencing) or genome sizes (for shotgun sequencing), so the 'actual' ratios vary slightly between the two analyses even though the DNA concentrations are identical. Genus-level taxonomic bar charts by (16S and WGS analysis) show the average composition observed for each DNA mixture (Figure SI-8).

Discussion

The MSC represented the third in a series of community challenges of increasing complexity hosted by Janssen's Human Microbiome Institute (JHMI) as an effort to improve the overall quality of microbiome MGS measurements. This study was designed and implemented through a collaborative effort that included the Janssen Human Microbiome Institute (JHMI), The BioCollective, LLC (TBC), DNAGenotek, DNANexus, and the National Institute of Standards and

Technology (NIST) which serves as the National Metrology Institute for the U.S. These organizations in turn represent biopharmaceutical companies, biotechnology companies, data analytics companies, and Federal Government laboratories, all of whom have a vested interest in reliable and comparable microbiome measurements. The goal of the MSC was to capture the diversity of protocols for MGS-based microbiome measurements in an effort to begin to elucidate the impact of these methodological variables on the resulting taxonomic profiles and guide the development of future reference materials.

The MGS workflow required for microbiome analyses is complex. Therefore, designing an interlaboratory study that includes a multitude of the methodological variables and assesses their effect on the results is an ambitious project. Several teams have sought to address the question of methodological bias and variability over the years. [26-28, 31-33, 39, 40] These investigations have taken a variety of approaches from prescribing locked-down SOPs and analyzing specific samples to more open-ended data collection. The interlaboratory study presented herein specified seven samples for analysis (5 different stool samples and 2 DNA mixtures) while intentionally leaving protocol choices up the participating labs, both to sample a diverse set of methodological parameters as well as to survey common methodological choices.

The design and implementation of this project can be broken into three major areas: (1) reference material selection and production, (2) capturing metadata and MGS raw data, and (3) comparing results between participating laboratories.

Reference Material Production

One of the first decisions was the identification of reference material(s) to include. There are two primary types of materials that have been used for this type of study: (1) biologically derived microbiome samples and (2) mock communities. Both types of materials were included in the current investigation because they are useful in different ways for comparing between diverse analytical workflows.

For biologically derived microbiome reference materials, a natural community (e.g. sludge, soil, fecal material) is collected, homogenized, and aliquoted. Previous interlaboratory studies have used these homogenized real-world materials [31, 39]; however, the number of units needed and the associated costs of a large-scale study are often prohibitive. Further, while biologically-derived materials represent the complexity and diversity of real-world samples, they currently lack ground-truth value assignments (e.g. actual taxonomic abundances) due to a lack of unbiased analytical methods (e.g., DNA extraction, PCR amplification) and the inherent ambiguity associated with microbial taxonomy that hinders our ability to define clear measurands (e.g., Escherichia coli vs. Shigella, or the recent reclassification of Lactobacillus into 23 novel genera). [48] The addition of allochthonous bacteria (“spike-ins”) at consistent abundances into biologically-derived materials can provide some ground truth values to facilitate the assessment of MGS measurements.

Nevertheless, these biologically-derived materials remain useful for comparing methods and assessing measurement precision within individual laboratories and across different laboratories. In the current study, five stool samples were selected based on their dissimilarity

379 from one another among a constellation of potential stool donors (Figure 2), with the intention
380 of representing the variability of naturally-occurring samples. Preliminary in-house analysis of
381 individual aliquots demonstrated (Figure 3) that the material collection and preparation
382 resulted in samples with reliable between-aliquot homogeneity, even given the inherently
383 inhomogeneous starting point of multiple donations of human stool.

384 Mock community reference materials are laboratory-prepared mixtures of defined constituents
385 (typically DNA from individually cultured bacteria; sometimes mixtures of whole cells) at
386 specified amounts. Thus, these materials are useful as 'ground truth' for analysis workflows,
387 allowing quantitative assessment of analytical performance (e.g., accuracy, bias, precision, etc.).
388 However, these mock community materials are inherently non-biomimetic of actual
389 microbiome samples (e.g., feces, soil, etc), namely due to their low complexity and the absence
390 of a matrix-effect, which can limit their utility for assessing analysis workflows. [26] By including
391 both types of reference materials (5x stool samples and 2x mock communities) in the MSC, we
392 sought to include the benefits of both, using the biologically-derived materials to assess MGS
393 measurement variability between different protocols, and using the DNA mixtures to assess
394 MGS bias with respect to ground truth values.

395 **Capturing Metadata**

396 The universe of discrete MGS methodologies is quite large. Preliminary projections during
397 project planning estimated that several hundred samples would be needed to fully explore this
398 methodological space. Thus, the MSC set-out to host an international interlaboratory study on
399 an unprecedented scale. 700 units of reference material were prepared and made available
400 free-of-charge, where each unit consisted of 5 distinct, biologically-derived human fecal
401 microbiome samples and 2 DNA mixtures (mock communities). To our knowledge, there has
402 never been an MGS interlab study designed on such a massive scale. However, during the 19-
403 month marketing campaign, only ~100 units were distributed. Further, from these recipients,
404 only 44 sets of raw sequencing data and metadata were submitted (Table 1), limiting the
405 statistical power of the resulting analyses. Nevertheless, the unused units remain currently
406 available from The BioCollective, allowing interested researchers to analyze with their own
407 methods using the same samples that have been characterized and reported on here.

408 Alongside the raw sequencing data submitted, participating laboratories filled out a metadata
409 questionnaire (available in supplemental file 1) with ~100 discrete questions about the
410 methods employed, most of which allowed selection from drop-down options describing the
411 most common methodological choices. However, it must be noted that even these in-depth
412 options were not sufficient to encompass all experimental possibilities, and many metadata
413 selections represented 'Other' or 'Internal Method' options. And, of course, the number of
414 potential methodologies continues to expand as new techniques are developed or made
415 commercially available. It was also apparent within the submitted metadata that the observed
416 methodological choices were not randomly distributed. There was no effort made in this
417 investigation to encourage exploration of a diverse set of methodologies, and groups tended to
418 cluster around common methods. The resulting metadata reflect the most employed methods
419 during the timeframe of this study (Figure. 1). For instance: nearly half of participants analyzing
420 samples by 16S reported using the same DNA extraction kit (there were ~15 other pre-

421 identified options, as well as 'In-house' and 'other' possibilities); and only 2 labs (~4%) used
422 non-Illumina sequencing platforms.

423 **Comparing Results Between Laboratories**

424 To help assess the impact of methodological choices in the context of compositionally-sensitive
425 MGS measurements, we focused here on ratios between Phyla (e.g., the
426 Firmicutes:Bacteroidetes ratio: Figure 5) instead of the raw relative abundances [46]. By using
427 this strategy to remove the compositional dependence of MGS results, common statistical tools
428 (e.g., mean, standard deviation, confidence intervals) could be directly applied. However, it
429 must be noted that the Firmicutes:Bacteroidetes ratio only reveals the impact of particular
430 methodological choices on the tested phyla (Firmicutes and Bacteroidetes). Thus, a
431 methodological choice that only impacted Proteobacteria, as well as one that affected
432 Firmicutes and Bacteroidetes similarly, would not be noted herein. Nevertheless, significant
433 variability in the Firmicutes:Bacteroidetes ratio was observed (Figure 5) both between samples
434 (presumably due to real differences between the samples) and between participating
435 laboratories (presumably due to differences in measurement methodology).

436 When comparing between methodologies, the most basic experimental choice is between 16S
437 and WGS, and this choice had further implications for how subsequent steps were performed
438 (e.g., PCR conditions, library prep, sequencing depth, bioinformatic analysis). Thus, we first
439 compared the Firmicutes:Bacteroidetes ratio between analysis methods (Figure 5). In this case,
440 it turned out that the most basic choice of how to analyze samples had a statistically significant
441 effect (Figure 6). Analysis of each of the stool samples individually (Figure SI-3) or statistical
442 analysis using alpha diversity instead of the Firmicutes:Bacteroidetes ratio (Figure SI-4)
443 confirmed the significant impact of analysis strategy on observed results. Practically, this raises
444 real concerns about the comparability of data results between laboratories whose analyses
445 differ between 16S and WGS analysis. More generally, researchers should use utmost caution
446 when trying to compare between data sets collected using divergent experimental methods.

447 Within the data collected for the MSC, the significant effect observed for the choice of analysis
448 strategy had the specific implication of further limiting statistical power (e.g., of the 44
449 participating labs, 30 reported 16S results and 14 reported WGS results). Nevertheless, the
450 observed effects of other methodological choices could be similarly assessed for 16S (Figure 7)
451 or WGS (Figure SI-4) results. Interestingly, while the statistical power was limited in this study,
452 some methodologies still appeared to have either large effect sizes or large impacts on
453 variability/precision. While it is tempting to draw firm conclusions from the current
454 investigation, caution is warranted due to the limited sample sizes. Instead, it is hoped that this
455 investigation will help guide further investigations.

456 **'Spike-in' organisms**

457 During production of the stool samples, two exogeneous, 'spike-in', whole cell bacterial strains
458 were included, *A. fischeri* and *L. xyli*. Both strains are typically absent in human stool. With the
459 addition of 10^8 cells/mL, each organism was expected to comprise approximately 1 % of the
460 total stool relative abundance, providing sufficient signal for identification without significantly
461 affecting the overall sample profile. Unfortunately, while this expectation proved accurate for
462 *A. fischeri*, *L. xyli* was not identified in any the of 16S MGS results and was only observed at a

463 very low relative abundance by WGS (Figure SI-7). This absence or low-level detection could be
464 the result of a number of sources including lack of representation in the databases, bias in the
465 DNA extraction of *L. xyli,* or the amount of *L. xyli* added to the samples. However, multiple
466 coauthors were individually able to reliably detect *L. xyli* using alternate bioinformatic pipelines,
467 so it is likely that its limited detection in this dataset reflects a shortcoming in the reference
468 database used [data not shown and manuscript in preparation]. This explanation is also
469 supported by the observation that for WGS analyses, the variability of the ratio of spike-in
470 relative abundances between samples was somewhat improved among the labs with the
471 deepest sequencing results (Figure SI-2). It is worth noting that all raw fastq data submitted
472 through the Mosaic Standards Challenge has been archived and made publicly available for the
473 exploration of alternate bioinformatic methods.

474 The inability to reliably detect *L. xyli* within the framework of this project impacts our ability to
475 accurately and confidently use *A. fischeri* as well since observing a constant ratio between the
476 two spike-in organisms is fundamental to trusting their utility (Figure SI-7). Nevertheless, key
477 considerations were identified for future experimental design and implementation of internal,
478 spike-in controls. First, the strain should normally be absent in the sample, but still identifiable
479 by the analysis/database used. This can be tricky because databases often focus on the
480 organisms commonly encountered in each type of sample, and because the users of
481 bioinformatic pipelines may not have easy access to the underlying reference databases at the
482 time of analysis. Second, spike-in abundance should be sufficiently high that it can withstand
483 potential losses in the processing and still be identified, while not significantly compromising
484 the fraction of sample reads allocated the organisms native to each sample. This is in turn
485 complicated by the dependence of the observed relative abundance of any spike-in organism
486 on the MGS methods to be employed and their potential for bias with respect to each spike-in
487 organism. And third, the inclusion of additional spike-in organisms (e.g., 3-4 spike-ins total)
488 should be considered when MGS workflows have not been identified and tested a priori. This
489 provides redundancy to accommodate wide ranges of MGS methodologies and biases. In this
490 study, the inclusion of additional organisms could have avoided the problematic absence of *L.*
491 *xyli* in the reference database.

492 **DNA Mock Communities**

493 The DNA mixtures provided the ground-truth component in this study. Here, measurement bias
494 was observed as a disagreement between the actual ratios (black bars show the 99 %
495 confidence interval) and observed ratios (red and blue points) in Figure 8. This bias depends on
496 both the particular taxa analyzed, as well as the methods employed (16S vs. WGS is broken out
497 here). Interestingly, even where there was consensus between participating labs (i.e., a narrow
498 boxplot indicating strong consensus), substantial bias was still observed (low accuracy). The
499 consensus between participating labs is particularly apparent in the WGS analysis of the equi-
500 genomic DNA mixtures (upper right panel, Figure 8) suggesting some systematic bias affecting
501 each lab. Of note, these mixtures were comprised of genomic DNA from a prototype reference
502 material. Since the time of the MSC, NIST has completed a full characterization of DNA from 19
503 bacterial strains; NIST Reference Material, RM 8376, is now available for researchers to
504 construct their own DNA based mock communities [49].

505 **<u>Conclusion</u>**

506 From 2017 to 2020, the MSC provided a set of biologically-derived and mock community
507 microbiome samples, at no charge, to any interested MGS research group in an effort to
508 identify the extent of methodological variability between researchers and assess its impact on
509 measured taxonomic profiles. 44 research groups submitted both raw MGS data and detailed
510 metadata about their in-house sample-handling protocols; although this represents a large
511 number of participating laboratories by most interlaboratory efforts, it remained statistically
512 limiting for the large number of metadata parameters (≈100) that were explored. Initial choices
513 about analysis strategy (i.e., amplicon vs. shotgun) significantly impacted the observed
514 Firmicutes:Bacteroidetes ratios across all samples. The null hypothesis of no significant effect
515 could not be ruled out for most methodological choices within this study, though some
516 appeared to have real effects on results (i.e., bias) or measurement precision (i.e., variability).
517 Thus, the results collated herein should help refine the scope of future assessments of
518 methodological choices. To this end, researchers at NIST have undertaken a pairwise approach
519 to systematically compare select steps within the metagenomic workflow (manuscript in
520 preparation). Additionally, through the inclusion of DNA mock communities with
521 independently-measured ground-truth abundances, we were able to assess the accuracy of
522 MGS measurements and observe significant and systematic measurement bias, even when
523 participating laboratories achieved similar results. Overall, the MSC effort has significantly
524 expanded our understanding of the impact of methodological choices on MGS measurement
525 results and precision.

526 **Methods[1]**

527 **Selection of Stool Donors**

528 A total of 5 donors were selected from a donor pool maintained by TBC. Figure 2 shows a Bray-
529 Curtis PCoA ordination plot the entire donor pool, including the 5 donors selected, based on
530 their gut microbiome composition. The 5 donors were selected based on the dissimilarities of
531 their microbiome composition (Figure 2).

532 **Sample Collection and Processing**

533 All stool samples were collected in accordance with TBC's Institutional Review Board protocol
534 and have been de-identified. The donors were provided with collection kits, and samples were
535 returned to the TBC via overnight shipping for processing. Upon receipt, the samples were
536 aseptically transferred to a zip-top bag for dispensing. The samples were stored at -80 °C in 30 g
537 aliquots until further processing. Multiple bowel movements were collected and pooled from
538 each donor. Material from each donor was processed individually (to avoid cross
539 contamination) and inside a biological safety cabinet. Using a Ninja blender, 150 g of fecal

1. Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose. The reference materials used in this study were not certified by NIST and are not official NIST Reference Materials.

540 material was combined with 150 g to 300 g of dry ice and homogenized into a fine powder. The
541 blender was loosely covered with a sterile lab tissue and placed in a -20 °C freezer overnight to
542 allow the remaining dry ice to sublime. For each sample, before the addition of OMNIgene
543 Stabilizing Solution (OGS), 50 g of neat powder was set aside and stored at -80 °C.
544 Approximately 90 g of stool powder was added to 750 mL of OGS. The solution was covered
545 and left to stir overnight at room temperature. The following morning, 1 mL aliquots were
546 prepared and stored at –80 °C.

547 **Addition of Spike-In Bacteria and Aliquoting of Samples**

548 Spike-in bacteria, *Aliivibrio fischeri* (formerly known as *Vibrio fischeri*, Gram negative) and
549 *Leifsonia xyli* (Gram positive), were grown to an approximate density of 10^8 CFU/mL and
550 10^9 CFU/mL, respectively. Cell concentration was confirmed via plate count and optical density.
551 The spike-in bacteria were concentrated by centrifugation, resuspended, and added to each
552 stool solution 1 hour prior to aliquoting to ensure thorough homogenization. Working in a
553 biological safety cabinet, the solution was aliquoted using wide-bore pipette tips into (800 to
554 850) aliquots. Final concentration of stool after addition of the spike-in was 100 mg/mL and
555 final concentration of each spike-in organism was 10^8 CFU/mL. Samples were stored at -80 °C
556 until distribution.

557 **Sample QC**

558 To assess the homogeneity of the stool samples, ten aliquots from each donor pool were
559 subjected to 16S rRNA amplicon sequencing and shotgun metagenomic sequencing. All sample
560 processing, DNA extraction, library preparation and sequencing steps were conducted at
561 CosmosID (Germantown, MD) using proprietary protocols. For the 16S sequence data, reads
562 were demultiplexed using split_libraries.py with default filtering parameters. 16S rRNA gene
563 sequences were then sorted based on sample ID using the QIIME script
564 *extract_seqs_by_sample_id.py.* Bacterial operational taxonomic units were selected using
565 *pick_open_reference_otus.py* workflow. 16S rRNA taxonomy was defined by ≥ 97 % similarity to
566 reference sequences using the *core_diversity_analyses.py script*. Alpha diversity, alpha
567 rarefaction curves, and taxonomy assignments were determined using the core_diversity.py
568 workflow. Data were rarefied to 100,000 sequences per sample to minimize the effect of
569 disparate sequence number on the results. Alpha diversity metrics were computed from the
570 average of 100 iterations from the alpha collated results. Microbiome features were quantified
571 from metagenome data using existing [Metaphlan2, HUMAnN2, etc.] and in-house pipelines to
572 identify strain-level taxonomic markers for all samples.

573 **DNA Mixtures**

574 Mixtures of purified genomic DNA from thirteen ATCC-derived strains were prepared in 1X TE
575 buffer at a final concentration of ≈100 ng/µL. The two mixtures were made by combining the
576 genomic DNA from the following bacterial strains: *Staphylococcus aureus* ATCC BAA 44,
577 *Staphylococcus aureus* ATCC 12600, *Pseudomonas aeruginosa* ATCC BAA 47, *Enterococcus*
578 *faecalis* ATCC 19433, *Salmonella enterica* ATCC 700720, *Salmonella enterica* ATCC 12324,
579 *Escherichia coli* ATCC 43895, *Staphylococcus epidermidis* ATCC 12228*, Klebsiella pneumoniae*
580 ATCC 13883, *Shigella sonnei* ATCC 25931, *Streptococcus pyogenes* ATCC 12344,
581 *Corynebacterium amycolatum* ATCC 49386. The individual genomic DNA components were part

582 of a prototype reference material and were not fully characterized at the time of the MSC.
583 Subsequent analysis revealed some of the materials were cross contaminated with other
584 components from the prototype materials including *Achromobacter xylosoxidans.* Mix A was
585 designed to be equi-genomic with calculated relative abundances by mass of each strain
586 ranging from ≈6.8 % to ≈10 %. Mix B was designed as a log-dilution of the genomes varying
587 across 3 orders of magnitude (from ≈0.01 % to ≈30 % by mass). For each mixture (A and B), we
588 prepared a single pool and then distributed across 700 aliquots where each contained
589 approximately 20 µL (2 µg) of DNA per aliquot. An average (across all participating laboratories)
590 relative abundance plot for each sample by amplicon or shotgun sequencing is included in the
591 supporting information (Figure SI-8). We performed digital droplet PCR (ddPCR) to measure the
592 absolute abundance as ground truth for the following species in the mixture: *Enterococcus*
593 *faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*. These
594 species were selected because they were taxonomically distinct within the mixtures at the
595 Genus level, facilitating MGS discrimination. Pairwise ratios of these abundances provided the
596 ground truth values depicted in Figure 8. The validated ddPCR assays were reported previously
597 [49].

598 **Interlaboratory Study Execution:**
599 Recruitment**:** Starting in the Spring of 2018, we launched a media campaign that targeted the
600 scientific community via social media and email blasts as an attempt to recruit a large and
601 international cohort of participants. After the MSC launched in May 2018, we continued the
602 outreach campaign via public speaking engagements at various international microbiology
603 conferences. We actively recruited volunteers up until January 2020 when the MSC officially
604 closed. MSC reference materials were shipped to any lab in the world, upon request, from May
605 2018 till January 2020.

606 Data Availability: The Mosaicbiome.com web portal was used during MSC to store, analyze,
607 visualize, and share all the raw data and metadata that was submitted by the MSC participants.
608 However, in the Spring of 2022, the site was discontinued due to the costs associated with data
609 storage and maintenance. Therefore, these data (fastq files and metadata summaries) have
610 been made available via https://data.nist.gov/od/id/mds2-2830.

611 Sample Availability: At the time of publication, many aliquots of the stool and DNA materials
612 generated through the Mosaic Standards Challenge still remain available from TBC.
613

614 **Taxonomic Profiling of Interlab Data**
615 All raw sequence data (fastq files) generated by interlab participants were downloaded from
616 the MosaicBiome web portal and subsequently analyzed via the CosmosID
617 (www.CosmosID.com) taxonomic classification tool using the CosmosID reference genome
618 databases (WGS version: 1.0.2; 16S version: 1.1.0). The MGS results (taxonomic profiles) for all
619 the MSC data are publicly available and can be found by visiting https://app.cosmosid.com and
620 following the directory structure: *Datasets -> Example_Datasets -> Mosaic_Microbiome*.

621 **Analysis of mosaic data results and methodological parameters**
622 A total of 50 datasets were received. Two datasets were dropped due to incomplete metadata
623 and an additional 4 labs were The MGS results (taxonomic profiles) and associated metadata

from all submitted data sets were analyzed using R. The raw data and code used for analysis and to generate the figures in this manuscript have been shared via https://data.nist.gov. [exact link TBD]

Acknowledgements

Funding for the production of the fecal reference materials and reference material shipping was generously provided by the Janssen Human Microbiome Institute (JMHI) .

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