Title:

A Sensitivity Analysis of Methodological Variables Associated with Microbiome Measurements

Authors:

Samuel P. Forry[1], Stephanie L. Servetas[1], Jennifer N. Dootz[1], Monique E. Hunter[1], Jason G. Kralj[1], James J. Filliben[2], Scott A. Jackson[1]

Affiliation:

1. Complex Microbial Systems Group, National Institute of Standards and Technology (NIST), Gaithersburg, MD
2. Multimodal Information Group, National Institute of Standards and Technology (NIST), Gaithersburg, MD

Abstract

The experimental methods employed during metagenomic sequencing analyses of microbiome samples significantly impact the resulting data and typically vary substantially between laboratories. In this study, a full factorial experimental design was used to compare the effects of a select set of methodological choices (sample, operator, lot, extraction kit, variable region, reference database) on the analysis of biologically diverse stool samples. For each parameter investigated, a main effect was calculated that allowed direct comparison both between methodological choices (bias effects) and between samples (real biological differences). Overall, methodological bias was found to be similar in magnitude to real biological differences, while also exhibiting significant variations between individual taxa, even between closely related genera. The quantified method biases were then used to computationally improve the comparability of datasets collected under substantially different protocols. This investigation demonstrates a framework for quantitatively assessing methodological choices that could be routinely

performed by individual laboratories to better understand their metagenomic sequencing workflows and to improve the scope of the datasets they produce.

Introduction

The democratization of access to next-generation sequencing (NGS) has made this technology the workhorse of modern microbiology. NGS-based metagenomic sequencing (MGS) enables analysis of the genomic content of microbial communities, reporting both the taxonomic identification and count of sequencing reads and even enumeration of constituent microbes. In recent years, MGS has increasingly found its way into the clinic and other regulated spaces such as quality control testing where the measurement results can have important consequences.(1-3) Despite widespread adoption of MGS capabilities, the analytical performance remains uncertain, particularly when comparing between protocols.(4)

While MGS measurements may appear straightforward, the results actually depend on a complex series of steps and sample manipulations, typically including: sample storage and transport, DNA extraction and purification, DNA fragmentation and barcoding, library preparation and NGS, bioinformatic analysis to identify and enumerate unique microbial signatures, and taxonomic assignment against a database, as well as additional bioinformatic analyses dependent on particular experimental designs and project goals. Further, within each of these steps, many methodological choices must be specified for the particular protocol employed. Each of these steps, as well as the individual methodological choices, can introduce bias and variability that then accumulate through the measurement process. (5-7) The methodological contributions of bias and variability can be visually depicted using an Ishikawa diagram (Figure 1).

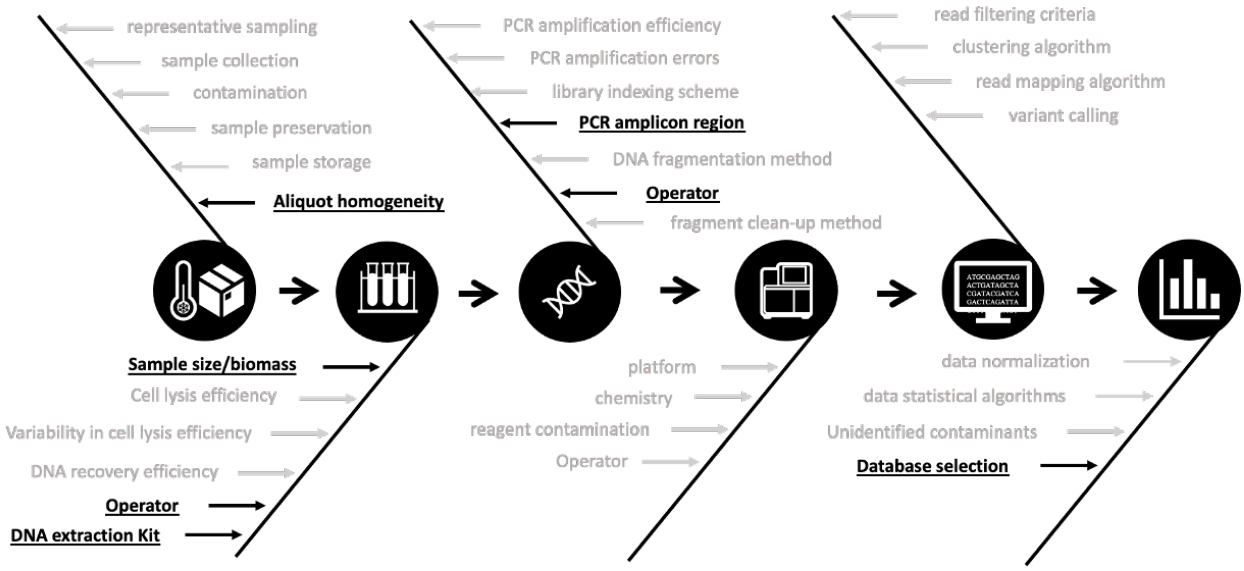


Figure 1 (link): ***Ishikawa diagram of bias associated with an amplicon metagenomic measurement workflow.*** *The central image depicts a typical metagenomic analysis: sample handling and storage, DNA extraction, sequencing library prep, next-generation sequencing, sequence data processing, and bioinformatic analysis. At each step, some of the various methodological choices that can introduce variability and bias are listed. Bold and underlined steps indicate methodological choices explicitly varied in the current study.*

In general, MGS datasets exhibit high precision (low variability) for locked-down workflows (8, 9), and this makes MGS particularly useful within a research project where protocols can be easily controlled. Given the myriad options for any single step (as well as a dynamic field where procedures continue to evolve quickly), it is unlikely that two different labs would implement the exact same measurement workflow from start to finish without substantial coordination. Hence, the lack of reproducibility between laboratories is perhaps not surprising even when the results are highly reproducible within a laboratory.(10) While there are limited opportunities through the MGS workflow for intermediate characterization of the impact of each methodological decision, there are experimental designs aimed at assessing the effect sizes of particular methodological decisions at the conclusion of the MGS

measurements.(11) One commonly used approach is a full factorial design, evaluating at least two specified options ('Level') for each tested methodological parameter.(12-14) This experimental design allows for a rigorous statistical assessment of the effect of individual parameters and provides quantitative data for direct comparisons.

We previously described the Mosaic Standards Challenge (MSC), an international interlaboratory study designed to assess the impact of methodological variability on MGS results.(15) The MSC employed five biologically distinct human fecal reference materials and a comprehensive standardized metadata reporting sheet that allowed participants to share exhaustive details about the protocols and methods used for analyzing the fecal materials. The MSC employed an "open protocol" design that allowed (and even encouraged) participants to follow divergent protocols as defined for their lab's routine MGS measurements. This strategy showcased the diversity of methodologies commonly employed across MGS measurement labs and highlighted the challenge of comparing MGS results between different protocols. In the current effort, we used the same five fecal samples from the MSC, but employed a fully specified experimental design where a small subset of methodological choices were systematically varied while the rest of the MGS measurement workflow was held constant.

The goal of this work was to demonstrate a rigorous experimental approach for evaluating bias in metagenomics workflows. Specifically, we used a full-factorial design (plus replication) for each fecal sample (n=5) to quantify the effects of different operators (n=2), reference material lot (n=2), DNA extraction kit (n=2), marker gene target (n=2), and reference database (n=3). The magnitude of effect for each selected methodological choice was quantified, and these main effects were compared between protocol choices and to the biological variability between fecal samples. Ratiometric analysis was employed to account for MGS measurement compositionality. In combination with spike-in species added to each fecal sample, this analysis allowed direct comparisons between samples. Among the

83 methodologies explored, the largest effects were observed for extraction kit selection, where the effect
84 varied substantially between individual taxa, even within taxonomic clades.

85 **Results and Discussion**

86 *Metagenomic Sequencing Methodologies and Experimental Design*

87 Using an Ishikawa diagram, a subset of parameters were selected (shown in bold and underlined) for
88 this initial proof-of-concept study (Figure 1). The experimental design included parameters that were
89 denoted 'biological' (i.e., actual biological differences between disparate stool samples) or
90 'methodological' (i.e., bias associated with the measurement protocol) to allow for direct comparison of
91 the variability from biological differences to the variability due to methodological differences. For this
92 study, 5 unique human fecal samples were selected representing the biological variability. For each
93 sample, the methodological parameters of operator (n = 2), lot (n = 2), extraction kit (n = 2), 16S variable
94 region (n = 2), database (n = 3), were chosen (with the indicated number of levels). Thus, 240 MGS
95 datasets (270 with replicates) were generated from 48 distinct workflows (Figure 2). The orange and
96 blue arrows in Figure 2 at the top and bottom arms of the study, respectively, represented orthogonal
97 workflows where unique methods were chosen for each parameter. The two orthogonal workflows
98 were replicated to assess precision, and the orange and blue lines were used here and in subsequent
99 figures to showcase data originating from the two completely distinct workflows.

100 This work represents a proof-of-concept and was intended to demonstrate a full factorial analysis of a
101 metagenomic sequencing workflow. Ideally, the implementation of a full-factorial experimental design
102 would specify 'high' and 'low' values for each selected parameter to bound the range of normal
103 experimental conditions. In the case of the metagenomics workflows evaluated here, the selected
104 experimental parameters of interest exhibited discrete, non-numerical, non-continuous levels (e.g.,
105 Operator, Lot, Extraction Kit), and the selected options (levels) may not bound the normal range of

experimental conditions. Herein, a rigorous statistical approach was demonstrated for evaluating bias in metagenomics workflows that others could utilize in their own laboratories with their particular protocols.

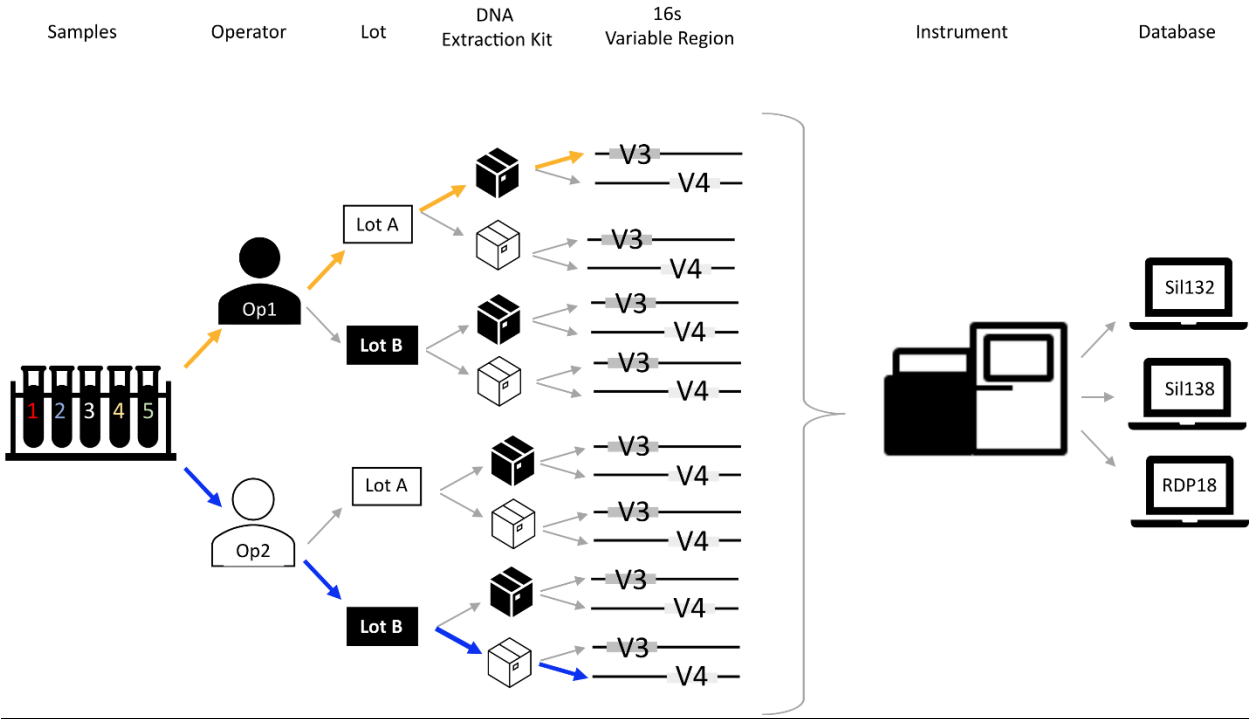
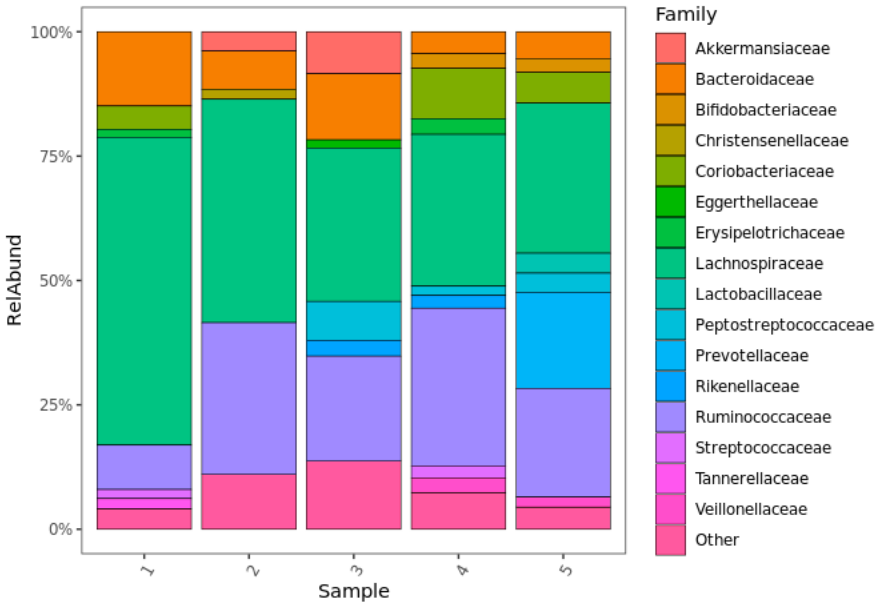


Figure 2 (link): ***Systematic experimental design enables quantitative bias assessment.*** *The graphic above illustrates the levels compared for each step of the metagenomic workflow. In total, 5 different human stool samples were handled by 2 operators, preparing samples from 2 lots, for MGS analysis using 2 DNA extraction kits and 2 variable regions, with taxonomic assignment coming from 3 databases. The top (orange) and bottom (blue) arms of the experimental design represent completely orthogonal protocol choices through all wet-bench operations, and these two protocols were replicated (2x) for each sample and database to explore reproducibility. This resulted in a total of 240 MGS data files (270 with replicates) that were analyzed.*

MGS: Compositional Analysis

A typical metagenomic analysis may include bar charts to show relative abundance and community members. As such, the composition of each sample for a single workflow was visualized in a relative abundance bar chart at the Genus taxonomic level (Figure 3A). The differences between stool samples were evident, and good reproducibility was observed between replicate analyses. For example, while *Lachnospiraceae* and *Ruminococcaceae* make up the plurality of each sample, their relative proportions vary, and some taxa (e.g., *Rikenellaceae, Prevotellaceae*, *Akkermansiaceae*) appeared variably present, occurring above the 1.5 % cutoff in some samples but not others. A comparison of MGS bar charts from alternate workflows showed that differences in sample composition could be correlated with methodological choices (Figure SI-1); however, using bar charts to assess methodological parameters can be difficult to implement systematically. While differences between samples can be readily observed, it is difficult to determine the magnitude of these differences or to correlate them with particular methodological decisions. Also, comparing the compositional changes between many individual bar charts proves to be cumbersome and challenging to automate.

Another common approach is to evaluate shifts in composition by calculating similarities (or dissimilarities such as Bray-Curtis) between MGS datasets and then generating a principal coordinate analysis (PCoA) plot from the resulting distance metrics (Figure 3B). In this approach, a single point was generated for each unique combination of sample and analysis workflow, and clusters of points were then correlated with methodological parameters. For example, with the data plotted in Figure 3B, data from a common sample (same colors) generally clustered, with some overlap and sub-clustering associated with methodological parameters (denoted with letter flags). To compare between specific methodological choices, additional faceting generally allowed for their relative impact to be assessed (Figure SI-2a-e). This pattern recognition approach facilitated the evaluation of multiple datasets simultaneously; however, the results remained mostly qualitative in nature, as the apparent significance of each evaluated parameter depended on the ordination conditions of that dataset.



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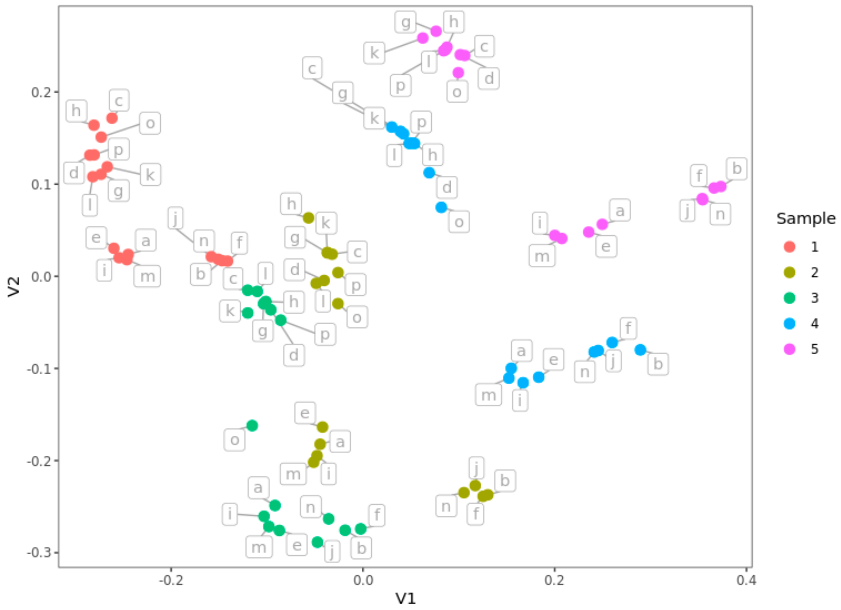


Symbol	Operator	Lot	ExtractionKit	VRegion
a	J	A	Q	V3
b	M	A	Q	V3
c	J	A	Z	V3
d	M	A	Z	V3
e	J	B	Q	V3
f	M	B	Q	V3
g	J	B	Z	V3
h	M	B	Z	V3
i	J	A	Q	V4
j	M	A	Q	V4
k	J	A	Z	V4
l	M	A	Z	V4
m	J	B	Q	V4
n	M	B	Q	V4
o	J	B	Z	V4
p	M	B	Z	V4

Figure 3: ***(a) Bar charts and (b) PcoA plots from 5 human stool samples.*** *(3A) Family level relative abundance bar charts show the compositional diversity among 5 human stool samples for a single workflow. For this plot, Families present at <1.5% relative abundance are grouped as "Other". Correlation between the observed compositions and specified protocol choices (see Figure SI-1) can help identify particularly sensitive steps in an MGS workflow. (3B) Bray-Curtis*

dissimilarities can also be used to identify sensitive MGS protocol choices. Points are colored here to indicate fecal sample and letter flags show additional protocol choices for each dataset, as denoted in the table. Further clustering (see Figure SI-2) can be used to rank protocol choices with respect to their impact on the observed composition. Though these two plots are conventionally used, they are limited in their utility to identify and address biases.

MGS: Ratiometric Analysis

An additional challenge when comparing MGS results between samples is the compositional nature of the datasets. That is, the measured relative abundance value for any taxa depended on both its actual abundance in the sample and on the sum of the observed abundances for all other taxa present in that sample. An alternative approach for comparing MGS results has focused on ratiometric comparisons, and these ratios of taxa have been shown to be *independent* of sample composition. (5, 8, 16-18) Whereas the initial relative abundance values could be misleading, ratios of relative abundance between two taxa within a sample have been shown to be more reliable, varying with just the actual taxa abundances and measurement biases.(8)

This concept has been implemented previously with native taxa, such as calculating the *Firmicutes:Bacteroidetes* (F:B) ratios (among others), with varying levels of success (10, 19-21). To demonstrate ratiometric analysis, we examined the F:B ratio within our experimental design (Figure 4). The F:B ratio provided a single calculated value for each unique combination of stool sample and experimental workflows (black points), with the samples analyzed under identical workflows connected by light grey lines. For reference, the two completely orthogonal workflows are colored orange and blue (and match the top and bottom arms of the experimental design in Figure 2).

As expected, due to real variation in the actual F:B ratio between biologically distinct samples, similar trends ('M' shape) were observed across samples for each of the different protocols evaluated. A

Kruskal-Wallis Test confirmed that there was a statistically significant difference in F:B ratio between the stool samples ($\chi^2(4) = 77.8$, p = 1e-15). However, within each sample, a greater than 4-fold difference in F:B ratio was observed between different workflows, reflecting the impact of methodological choices on the observed F:B ratio. Indeed, this workflow-dependent effect (bias) was at least as significant as the biological differences observed between distinct stool samples. In a rapidly evolving field like MGS, this kind of workflow-dependent measurement bias significantly complicates comparisons between studies.

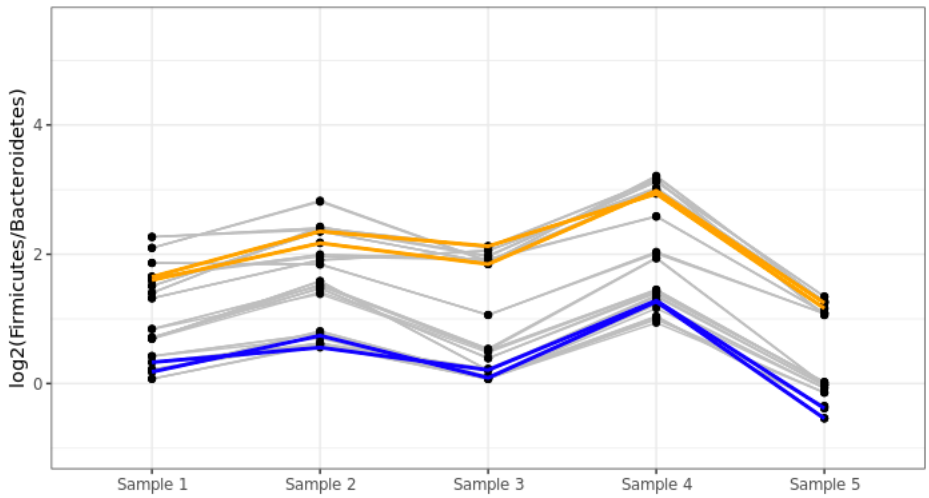


Figure 4: ***Impact of methodological parameters on the Firmicutes:Bacteroidetes ratio.*** *The Firmicutes:Bacteroidetes ratio was determined for each sample (black points) under every combination of the methodological parameters investigated in this study (48 combinations for each of the 5 samples). A grey line connects the dots between fecal samples processed using the same protocol; dots and lines overlapped for protocols where the varied parameters didn't change the observed Firmicutes:Bacteroidetes ratio. The blue and orange lines denote the results from the top and bottom legs of our experimental design flowchart (shown in Figure 2), respectively, and represent orthogonal choices for every methodological parameter (operator, lot, DNA extraction kit, variable region, database). There are two blue and two orange lines, as*

each of these workflows were run in duplicate to demonstrate reproducibility when all parameters are held consistent.

MGS: Internal Standards

Given that native taxa are expected to vary naturally between the stool samples, as observed here with Firmicutes and Bacteroidetes, an alternative approach for ratiometric analysis is to add exogenous strains (spike-ins) to serve as internal controls. In this study, two strains were uniformly added during the batch production of these stool samples. *Leifsonia xyli* (Gram positive) and *Aliivibrio fischeri* (formerly known as *Vibrio fischeri*, Gram negative), a plant pathogen and marine organism, respectively, were selected as they are unlikely to be found in human stool samples and could therefore be uniquely identified taxonomically. Unlike the native taxa, these spike-in organisms were added at consistent abundances across all stool samples.

Similar to the F:B ratio plotted in Figure 4, the *Leifsonia*:*Aliivibrio* (L:A) ratio was calculated for each unique combination of stool sample and experimental protocol (Figure 5). As before, a high degree of bias was observed between individual workflows (vertical distribution of points for each sample). However, unlike the previous analysis, the L:A ratio did not exhibit trends across samples ('M' shape from Figure 4). Supporting this lack of biological variability, a Kruskal-Wallis Test failed to find a statistically significant difference in L:A ratio between the stool samples ($\chi^2(4) = 4.8$, $p = 0.31$). Instead, observed variations in the L:A ratio between samples analyzed with a common workflow (i.e., following a single line trace) were attributed to measurement variability. Considering the differences in the relative abundances of *Firmicutes* and *Bacteroidetes* (≈88 %) versus *Leifsonia* and *Aliivibrio* (≈0.6 %), the increased measurement variability observed in Figure 4 was unsurprising. Since the spike-in organism abundances were detected consistently between samples, we concluded that either of these spike in organisms were suitable for use as internal standards for ratiometric analyses. For the purposes of

demonstrating ratiometric analysis we chose to use *Leifsonia*. As such, comparing the *Bacteroidetes:Leifsonia* ratio between different samples handled under a constant workflow allowed actual changes in Bacteroidetes to be quantified independent of any other native taxa.

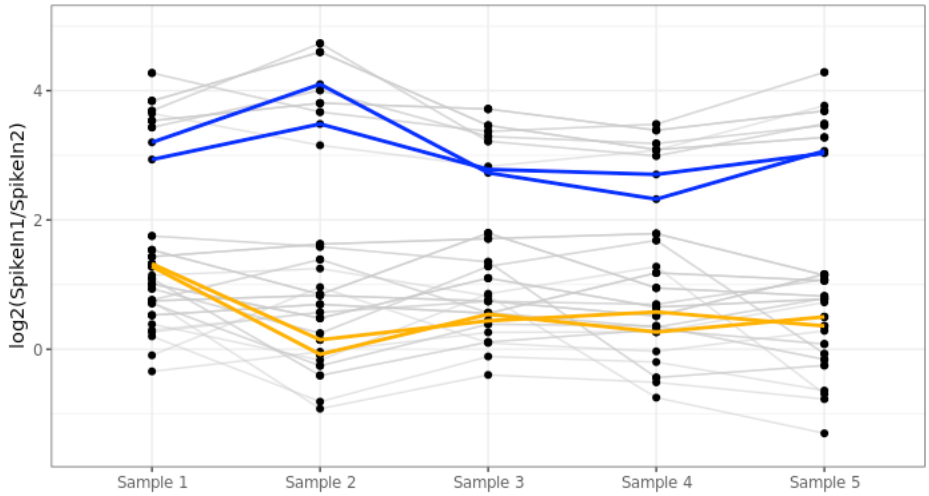


Figure 5: ***Analysis of internal standards added uniformly to all stool samples.*** *Two exogenous organisms, Aliivibrio and Leifsonia, were added as internal standards to the stool samples. The ratio of Aliivibrio:Leifsonia was calculated for each sample (black points) under every combination of the methodological parameters investigated in this study (48 combinations), as described in Figure 4. A grey line connects the dots between fecal samples processed using the same protocol; dots and lines overlapped for protocols where the varied parameters didn't change the observed Aliivibrio:Leifsonia ratio. The blue and orange lines denote the results from the top and bottom legs of our experimental design flowchart (shown in Figure 2), respectively, and represent orthogonal choices for every methodological parameter (operator, lot, DNA extraction kit, variable region, database). There are two blue and two orange lines, as each of these workflows were run in duplicate to demonstrate reproducibility when all parameters are held consistent.*

Quantifying Parameter Effects

In Figure 5, the systematically varied workflows resulted in significant variation in the ratiometric MGS results within each sample. The full-factorial experimental design (Figure 2) was structured to balance each decision point in the workflow and facilitate independent characterization of each methodological choice. A Parameter Effect was calculated for each evaluated step in the workflow (i.e., parameter) and the method selected (i.e., level) by calculating the fold change of ratiometric results from workflows using the specified method versus the average results across all workflows (Equation 1)

$$Parameter\ Effect_{(Parameter=Level)} = \log_2\left(\frac{GM(Ratio(Taxa{:}Lf)_{Parameter=Level})}{GM(Ratio(Taxa{:}Lf)_{All\ Levels})}\right) \quad (1)$$

where *GM* denotes the geometric mean. This calculated parameter effect allowed individual workflow steps and specific methodological choices to be directly compared.(11, 12)

As expected, when the Parameter Effects for the ratio of the two internal standard taxa were calculated, (Figure SI-3) no significant effect was measured between samples due to the even amount of the internal standards added to each sample; however, several of the methodological parameters did exhibit statistically significant Parameter Effects for the ratio of the two internal standards. It was important to note that the Parameter Effect calculation (Equation 1) was based on ratios with the internal standard and could not attribute observed effects to either of the ratioed taxa individually. When we subsequently considered taxa native to the stool, a large Parameter Effect denoted a large bias differential between the taxa-of-interest and the internal standard, while a small or negligible Parameter Effect indicated a similar response between the taxa-of-interest and the internal standard.

The Parameter Effect calculations for the phyla Bacteroidetes revealed significant effects for several parameters (Figure 6), additionally the differences between biological samples (e.g. aliquots) were also generally significant for Bacteroidetes. One benefit of the Parameter Effect calculation in the context of

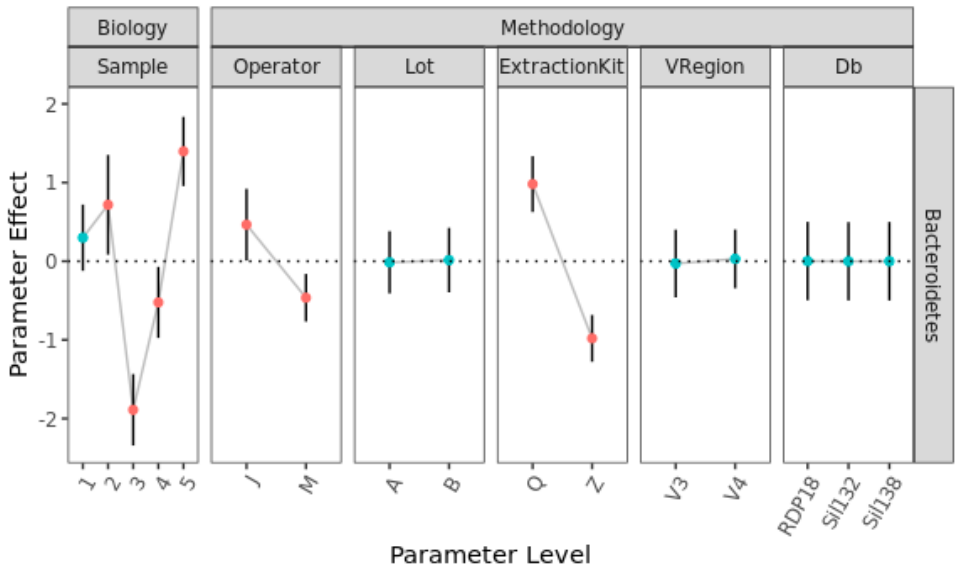
a full-factorial design was its ability to quantify the effects of methodological parameters even in the context of biological variations. This was confirmed by calculating the Parameter Effects for *Bacteroidetes* within each stool sample individually (Figure SI-4). The within-sample Parameter Effects matched the aggregate Parameter Effects, albeit with a larger (less precise) confidence interval due to the smaller number of samples. In the aggregate Parameter Effects calculation, the extraction kit and operator parameters exhibited statistically significant effects, while the parameters of lot, 16S variable region, and database did not exhibit significant effects.

The Parameter Effects for Extraction Kit were similar in magnitude to the real biological differences between samples, potentially complicating comparisons between samples that were analyzed using different methods. For example, comparing stool samples 4 and 5 using Extraction Kit Z, while holding all other methods constant, revealed an actual difference in *Bacteroidetes:Leifsonia* ratio (B:L) of 4.1-fold. However, evaluating only sample 4 using Extraction Kits Q and Z revealed an apparent difference in B:L of 4.4-fold, just due to the methodological difference. When the measured B:L was compared between an analysis of stool sample 4 using extraction kit Q and an analysis of stool sample 5 using extraction kit Z, the results were essentially identical (1.05-fold difference) because the effects of the biological and methodological differences were of similar magnitude and in opposite directions. Alternately, analyzing sample 4 with extraction kit Z and sample 5 with extraction kit Q yielded an apparent change in B:L of 10.3-fold because the biological and methodological differences compounded.

In addition to Bacteroidetes, Parameter Effects were calculated for multiple independent phyla across the 5 stool samples (Figure SI-5). As expected for diverse stool samples, the native phyla varied in different ways between the five fecal samples, reflecting their disparate biological compositions. However, the calculated Parameter Effects for methodological parameters also varied significantly between phyla. For instance, Proteobacteria and Bacteroidetes exhibited significant Parameter Effects for Extraction Kit, while Actinobacteria and Firmicutes did not exhibit significant Parameter Effects for

276 Extraction Kit (Figure SI-5). It was notable (while not unexpected) that these calculated Parameter

277 Effects were taxa specific.



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279 *Figure 6*: ***Quantitative comparison between methodological parameters****. The*

280 *Bacteroidetes:Leifsonia ratio was calculated for each dataset to enable comparison between*

281 *protocols. The effect of each parameter was calculated by dividing the average ratio for all*

282 *datasets at the denoted parameter level by the average ratio across all parameter levels, as*

283 *shown in Equation 1. This parameter effect was plotted as a fold change on a log2 scale, such*

284 *that the horizontal line at 0 denotes the null hypothesis of no effect. The magnitude of the effect*

285 *of protocol choices (e.g., Extraction Kit) can be directly compared between parameters and*

286 *parameter levels. Data error bars showed 99 % confidence intervals, and the points that are*

287 *statistically significantly different from the mean ($p<0.01$) were colored red.*

288 *Quantifying Parameter Effects: Selected genera*

289 While the analyses of native microbes presented thus far have focused on the highest taxonomic

290 classification (i.e., phyla), much published research has linked beneficial or deleterious biological

291 outcomes with more specific taxonomic clades.(22) Thus, we calculated Parameter Effects for a panel of
292 genera previously posited as having particular interest for gut health and representing multiple genera
293 within specified phyla (Figure 7). Overall, the methodological factors of lot and variable region did not
294 exhibit significant Parameter Effects for any of the genera evaluated here (and have been omitted from
295 the plot), while the factors: Operator, Extraction Kit and Database were sometimes significant.

296 Interestingly, while the two genera within the *Actinobacteria* phyla exhibited similar responses to the
297 methodologies evaluated in this study, this was not the case within the *Firmicutes* or *Bacteroidetes*
298 phyla. Within the *Firmicutes*, the *Faecalibacterium* genus was preferentially enriched by one extraction
299 kit and exhibited no Parameter Effect for operator, while the *Blautia* and *Anaerostipes* genera were
300 preferentially enriched by the other extraction kit and exhibited significant Parameter Effects for
301 operator. Within *Bacteroidetes*, the *Alistipes* and *Bacteroides* genera exhibited similar Parameter Effects
302 for extraction kit, but differed in the Parameter Effects for operator and database. These variations in
303 the Parameter Effects within Phyla demonstrated significant phenotypic heterogeneity at the genus
304 level and underscored the difficulty in identifying reference organisms as potential surrogates for other
305 members within a taxonomic grouping. To further demonstrate the variation in Parameter Effect an
306 expanded view of each taxonomic level (Phylum, Class, Order, Family) for the genera in Figure 7 is
307 present in Figure S1-6. In some cases, trends stayed the same as you move down the taxonomic
308 hierarchy (Figure SI-6e, operator) while in others significant differences were only observed at high
309 taxonomic levels (Figure SI-6f, operator) or low taxonomic levels (Figure SI-6f, Extraction Kit). The scope
310 of the current effort was limited to the specific parameters varied and the parameter levels employed;
311 however, similar conclusions (i.e., taxa-specific Parameter Effects that can vary substantially within
312 taxonomic groupings) should be expected for other MGS protocols.

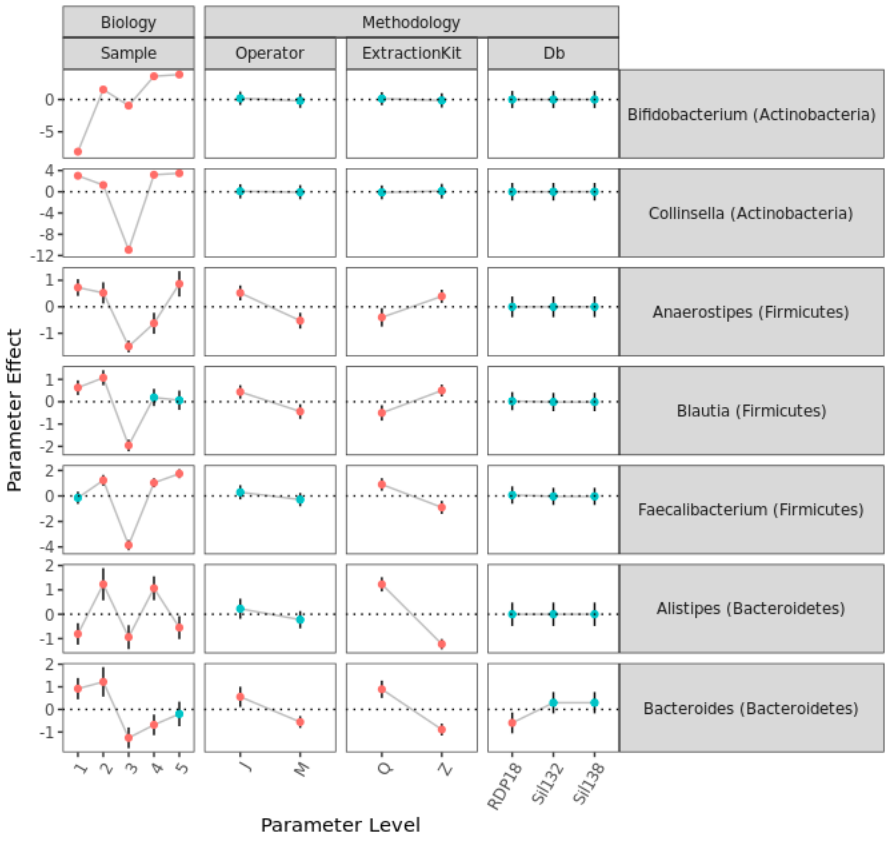


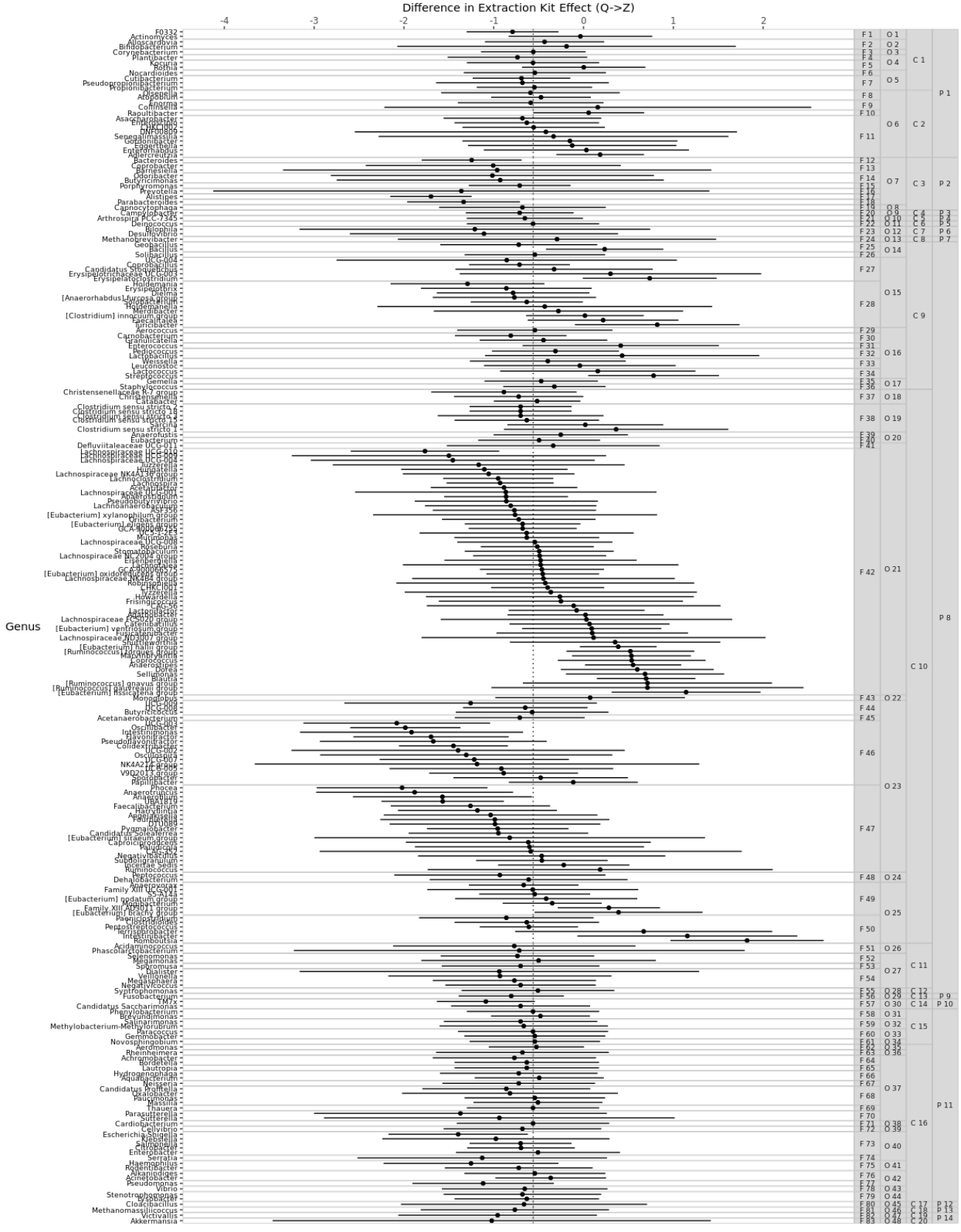
Figure 7: ***The effect of methodological parameter choices impacts the quantitation of some Genera associated in the literature with the health of the gut microbiome.*** *Like Figure 6, parameter effect was calculated for the ratio of various taxa of interest to an internal control (Leifsonia). The parameter effect was calculated by dividing the average results for the specified parameter level for the specified ratio by the average results across all factor levels for the specified ratio. Data error bars show a 99 % confidence interval, and statistically significant points ($p < 0.01$) are colored red. The effects broken out by taxonomic levels are plotted in SI Figures 6a-6g.*

Quantifying Parameter Effects: All Taxa

Using ratios with the internal standard, we calculated Parameter Effects for each individual taxa native to the fecal samples. The methodological parameter which generally exhibited the most significant

effect was Extraction Kit, and we focused on that Parameter Effect to assess the degree of variation between taxa (Figure 8). Additionally, discrepancies in taxa names between databases or even database versions made systematic comparisons of individual taxa challenging, so this quantification of the Parameter Effect for extraction kit was performed within the Silva 138 database.

For some taxa, such as those in the family Clostridiaceae (Figure 8, F38), there appeared to be little or no effect of extraction kit, with all 99% confidence intervals overlapping with the mean and no statistical difference between the 6 genera. In comparison, the Peptostreptococcaceae family (Figure 8, F50) exhibits a wide range of extraction kit effects (and statistically significant differences) between the 6 genera. In general, families with a greater number of genera present in the sample showed a greater range of extraction kit effects. While this observation was not surprising, it was notable that even closely related taxa could be impacted differently by methodological choices such as extraction kit. Figure 8 would certainly look different in the details for other methodological choices or MGS workflows, but this demonstrated that in general the effects of methodological parameters (i.e, method bias) could vary substantially between taxa, even closely-related taxa, and cast doubt on the ability of well-characterized 'reference species' to serve as adequate surrogates.(23) This observed lack of consistency at the genera level has important implications for developing mock communities that will adequately stress a workflow (24). Instead, these data suggested that method bias for particular taxa-of-interest should be measured through experimental characterization of the protocols being considered.



P- Phylum	C- Class	O- Order	F- Family
1 Actinobacteria	1 Actinobacteria (Actinomycetota)	5 Propionibacteriales	7 Propionibacteriaceae
	2 Coriobacteriia	6 Coriobacteriales	11 Eggerthellaceae
8 Firmicutes	9 Bacilli	15 Erysipelotrichales	27 Erysipelatoclostridiaceae
			28 Erysipelotrichaceae
	10 Clostridia	18 Christensenellales	37 Christensenellaceae
		19 Clostridiales	38 Clostridiaceae
		21 Lachnospirales	42 Lachnospiraceae
		23 Oscillospirales	44 Butyricicoccaceae
			46 Oscillospiraceae
			47 Ruminococcaceae
		25 Peptostreptococcales-Tissierellales	49 Anaerovoracaceae
			50 Peptostreptococcaceae
	11 Negativicutes	27 Veillonellales-Selenomonadales	54 Veillonellaceae
11 Proteobacteria	16 Gammaproteobacteria	37 Burkholderiales	68 Oxalobacteraceae
		40 Enterobacterales	73 Enterobacteriaceae

Figure 8: ***The Effect of Extraction Kit on Different Genera.*** *For each genera in the fecal samples, the difference in the Parameter Effects of the two Extraction Kits ($Effect_{Kit=Z} - Effect_{Kit=Q}$) was calculated. (Leifsonia was used as the internal standard.) Error bars show a 99 % confidence interval. Genus name is given on the left hand of the figure, with corresponding Phylum, class, order, and family groupings on the right. The table below the figure provides the taxonomic key for all Families with ≥3 Genera, and the full taxonomic key (249 genera) is included in the Supporting Information (SI Table 1).*

Correcting for Methodological Bias

Mathematically, quantification of Parameter Effect provided a mechanism for achieving comparability between differing protocols.(8) The observed differences between protocols in the observed relative abundances of individual taxa (i.e., method bias) could be computationally brought into agreement by correcting for the taxa-specific Parameter Effect. To validate this experimentally, Genus-level Parameter Effects were calculated for the methodological parameters of extraction kit and operator. These two parameters were selected because they had previously exhibited significant Parameter Effects for multiple tested Genera. Additionally, the Parameter Effects were only calculated using data from a single stool sample to simulate the ability of this approach to improve MGS characterization of previously untested samples. Stool sample 3 was used to calculate the Parameter Effects because it exhibited the most unique Genera. Using the calculated Parameter Effects, the observed abundances of Genera in all five samples were then computationally corrected to account for the protocol employed (i.e., which extraction kit and operator were specified). The initial- and corrected- relative abundances were used to calculated Bray-Curtis Dissimilarities between samples across all protocols as seen in the principal coordinate analysis plot colored by Sample, with the variability between protocols approximated with a 95 % data ellipse (Figure 9). The effect of the computational correction was evident

in the reduced size of the data ellipses for each sample, demonstrating a significant decrease in the observed dissimilarities between data collected under differing protocols.

It was notable that the improved comparability (reduced method bias) between protocols was observed for all five stool samples, even samples 1, 2, 4, and 5 that were not included in the Parameter Effect calculations. The ellipse centroids did not shift with computational correction because the Parameter Effect calculation was centered around a fold-change of 0 (no effect), and the arms of the experimental design were evenly balanced. It should also be noted that this computational correction did not necessarily improve the resulting data with respect to the actual biological composition of the samples. Rather, the data have been made more comparable between protocols by accounting for their taxa-specific biases. In this way, the dissimilarities within the datasets for each sample were reduced. This result strongly supported the idea that MGS data collected under differing protocols could be harmonized through the systematic analysis of common samples (e.g., reference materials).

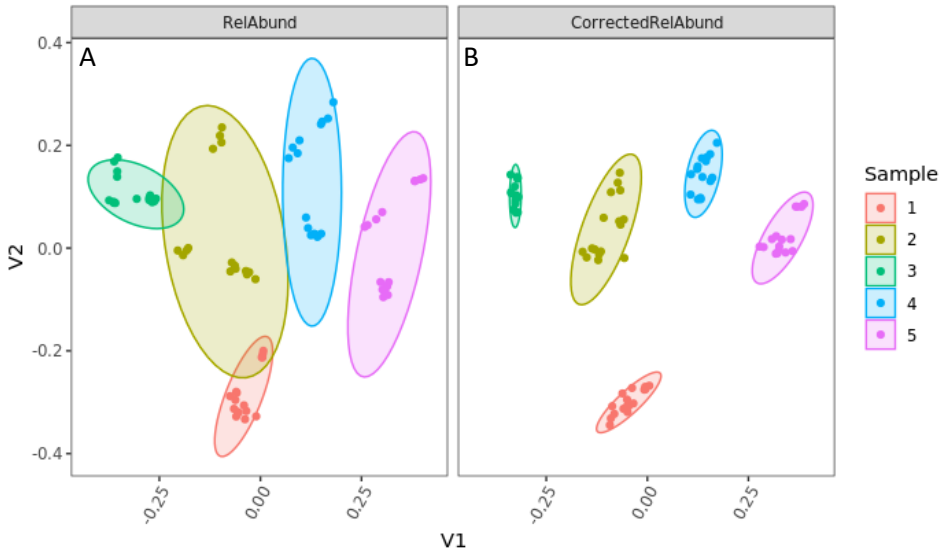


Figure 9: ***Correcting for Extraction Kit and Operator Biases.*** *Genus level Parameter Effects were calculated for Extraction Kit and Operator using data stool sample 3, and the Relative Abundances of individual Genera in all five samples were computationally corrected to account*

for the protocol used (i.e., which extraction kit and operator were specified). Bray-Curtis dissimilarities were calculated between all datasets and displayed on principal coordinate plots of (A) Relative Abundance or (B) corrected Relative Abundance, colored by fecal sample. 95 % data ellipses are shown to highlight the amount of dispersion observed between differing protocols for both conditions.

Conclusions

The impact of methodological variables on MGS results is generally well recognized. In the current manuscript we combined a full factorial experimental design strategy with ratiometric analyses to quantify the impact of specific methodological choices. When the F:B ratio was calculated for individual stool samples, a >4-fold divergence in results was observed between different protocols (Figure 4). Replicate analyses confirmed that MGS results were reproducible run-to-run, so the observed variations were attributed to bias associated with specific methodological differences in the analysis protocols. Furthermore, this methodological bias was similar in magnitude to the observed differences between the biologically distinct samples.

Using the full factorial design, the Parameter Effect could be calculated to directly compare between specific biological and methodological parameters (Figure 6). Among the 5 methodological parameters evaluated in the current investigation, extraction kit and operator were statistically significant, while stool lot, 16S variable region, and database were not statistically significant. The observation that extraction kit introduced significant measurement bias was not novel; however, the benefit of the full-factorial experimental design was that the Parameter Effect for each variable could be quantified and directly compared to the Parameter Effect associated with biological variations. For example, for extraction kits specifically, the observed Bacteroidetes measurement bias was sufficiently large to mask real differences between distinct biological samples. This observation raises questions about attempts to

406 compare MGS data collected under different protocols. Somewhat surprisingly, operator also yielded a
407 Parameter Effect that was weak but statistically significant and showed that human variability can still
408 impact results even with locked-down protocols and identical lab equipment.

409 By combining the full factorial design with ratiometric analysis against internal control organisms,
410 Parameter Effects could be calculated for individual taxa-of-interest native to the stool samples. For a
411 panel of 7 genera posited in the literature to be associated with specific gut-health outcomes, we
412 observed a large diversity of Parameter Effects (Figure 7), with some taxa exhibiting minimal
413 measurement bias for the methods evaluated here, while several others exhibiting significant bias
414 across multiple methodological decisions (Figure 7). Notably, Parameter Effects did not appear to group
415 by taxonomic clade (e.g., phylum) and this somewhat unexpected finding warrants further investigation.
416 Focusing specifically on the extraction kit, some related taxa exhibited similar Parameter Effects while
417 other closely related taxa (e.g., genera within a family) exhibited quite divergent Parameter Effects. This
418 diversity of methodological bias could have important implication for reference material development
419 (i.e., mock communities) where microbial mixtures often contain only 10s of strains. These findings
420 suggest that the generalizability of method performance based on a small subset of strains could be very
421 limited and justifies efforts to develop more complex mock communities and natural surrogate
422 reference materials that contain many more taxa and are compositionally similar to samples of interest.
423 Indeed, using genera-specific Parameter Effects calculated for one of the samples investigated here,
424 method bias between divergent protocols was significantly reduced for all samples.

425 The current report details some of the measurement challenges within the metagenomic space and
426 presents a method to systematically assess sources of bias. However, there are a few limitations worth
427 noting. As designed, this study only examined a small subset of methodological variables, while holding
428 all others constant. This enabled the full-factorial experimental design and allowed Parameter Effects to

be quantified for those variables. This study was not meant to serve as a comprehensive review of available kits, library preparations, NGS technology, databases, etc. largely due to a second limitation of the full factorial design: it is resource intensive and expensive. A significant amount of starting material was needed to ensure a homogenous sample supply, a notable challenge for clinical and archival samples that are routinely limited. As reagent costs and personnel time requirements quickly scale in increasing numbers of methodological variables, a partial-factorial experimental design may be sufficient in many cases. Nevertheless, as technology advances, the quantitative determination of the bias associated with specific changes to the workflow can make future results backward compatible.

In summary, we presented an experimental design and analysis strategy that enables direct quantitative comparison of different biological and methodological parameters. We encourage MGS research groups to consider characterizing the impact of their in-house protocol choices on their MGS results. As more labs seek to improve the comparability of their MGS results between divergent protocols, this approach provides a quantitative strategy for assessing methodological bias and shows how data collected under differing protocols can be harmonized by widespread analysis of a common sample (e.g., reference material).

Methods (Mosaic_Materials-and-methods.docx)

Samples and Sample Handling

Samples used in this study were previously described.(15) Briefly, five samples were generated from five different donors. Each sample was prepared by pooling and homogenizing 4 consecutive bowel movements from the donor, combining homogenized material with Omnigene Gut Solution, spiking with 10^8 CFU/mL of *Aliivibrio- fischeri* (Gram negative, formerly known as *Vibrio fischeri*) and *Leifsonia xyli* (Gram positive), and then aliquoting 1 mL aliquots. The final concentration of the material was 100mg/mL. Samples were received as a set of five, one sample from each donor (labeled 1-5). For this

study we ordered two sets (designated Lot A and Lot B) of the samples. Samples were stored at -80 °C until the experiments were conducted. On the day of the experiment, samples were vortexed vigorously and then 100 µL from each sample (1-5) was aliquoted into five 1.5-mL microcentrifuge tubes. This process was repeated for both lots. Operator 1 used three aliquots of each sample (1-5) from Lot A, and two aliquots from each sample (1-5) from Lot B. Operator 2 used two aliquots of each sample (1-5) from Lot A, and three aliquots from each sample (1-5) from Lot B.

DNA Extraction

Genomic DNA Extraction Kit Q:

Samples were extracted using two different methods. Method 1 was done with the QIAamp Fast DNA Stool Mini DNA prep protocol (Qiagen, Cat# 51504). Briefly, 1 mL of the Inhibit EX buffer was added to each of the samples and vortexed (Mo Bio Vortex – Genie 2 at max speed) for 1 minute. The samples were then centrifuged (Eppendorf Centrifuge 5417R) at 16,000×*g* for 1 minute to pellet the samples. A new 2 mL centrifuge tube was prepared containing 25 µL of proteinase K and 600 µL of the supernatant from the original tube was added to the tube containing proteinase K (supplied with kit). This was followed by the addition of 600 µL of Buffer AL; samples were vortexed for 15 seconds to mix. The samples were incubated for 10 minutes and 70 °C. After incubation, 600 µL of molecular biology grade ethanol (100 %, Sigma-Aldrich, E7023-1L) was added and vortexed. In 600 µL increments, the lysate was loaded onto the QIAamp spin column, and samples were then centrifuged 16,000×*g* for 1 minute. After centrifugation, columns were placed in new 2 mL collection tube and the flow-through was discarded. This process was repeated until all the lysate had been loaded onto the column. Next, the columns were washed first with 500 µL of Buffer AW1 (centrifuged 16,000×*g* for 1 minute), then 500 µL of Buffer AW2 (centrifuged at 16,000×*g* for 3 minutes). Flow-through was discarded and tubes replaced between each wash. To remove any remaining wash solution, columns were placed in a new collection tube and

centrifuged at 16,000×*g* for 3 minutes. Columns were then placed in 1.5 mL labeled microcentrifuge tubes and 100 μL of Buffer ATE was added to the center of the membrane. The samples incubated for 5 minutes and then centrifuged at 16,000×*g* for 1 minute to elute the DNA.

Genomic DNA Extraction Kit Z:

Method 2 was done with the ZR Fecal DNA miniprep (cat#D6010) from Zymo Research following the manufacturer protocol with minor modifications. Briefly, each sample was combined with lysis buffer and loaded onto the MoBio vortex Genie 2 with 2 mL tube adapter for 20 minutes at full speed. The lysis tube was centrifuged at 10,000×*g* for 1 minute. Then 400 μL of the lysate was added to the IV spin filter and centrifuged at 7,000×*g* for 1 minute. The process was repeated until all lysate had been passed through the spin filter. Filtrate was combined with 1,200 μL of the fecal DNA binding buffer and mixed well by pipetting. The mixture was transferred to the IIC column in 800 μL increments and centrifuged at 10,000×*g* for 1 minute. The supernatant was discarded. The bound DNA was washed and eluted in 150 μL of the elution buffer after incubation at room temperature for five minutes. The DNA was then transferred to the prepared IV-HRC spin filter and centrifuged at 8,000×*g* for three minutes.

Extracted DNA from both methods was quantified using DeNovix dsDNA High Sensitivity (Catalog number: KIT-DSDNA-HIGH-2) and analyzed on the DeNovix DS-11FX+ Spectrophotometer/ Fluorometer.

DNA Library Preparation

All PCR reactions were carried out using Kapa HiFi HotStart ready mix 2x Master Mix (KapaBiosystems, cat# 07958935001) in 0.2 mL thin wall PCR plate (Fisher Scientific, AB0800150). The V34 and V4 variable regions were chosen for 16S amplicon sequencing on the fecal samples. V34 (340F (5'→3') TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTACGGGNGGCWGCAG/ 806R (5'→3') GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT) and V4 (515F (5'→3') (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA/ 806R (5'→3')

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT) were ordered as pre-mixed primer pairs and include the Illumina handles (underlined) on the 5' end (RxnReady Primer Pools, IDT). The 16S PCR amplification was carried out using the following reaction mixture: 12.5 μL of Kapa HiFi HotStart 2x Master Mix, 1 μL of 10 μM V34 primer pool, 12 ng of extracted DNA, and nuclease free water to bring the final volume to 25 μL. Amplification was carried out using the following protocol: Initial denature (95.0°C – 3:00), 25 cycles of denature (98.0 °C – 0:20), annealing (55.0 °C – 0:15), elongation (72.0 °C – 0:20), and a final extension (72.0 °C – 1:00). The 16S reactions were purified using SPRIselect beads (Beckman Coulter, cat# B23318) to select for amplicons. The beads were added at a 0.8:1 ratio (20 μL of beads for 25 μL reaction), mixed by pipetting, and incubated on the bench for 1 minute then place on magnet for 1 minute. Without disturbing beads, liquid was discarded and while keeping the plate on magnet, 180 μL of 80 % molecular grade ethanol (Sigma-Aldrich, cat# E7023-1L) was added. After 30 seconds, ethanol, was removed carefully to not disturb the beads (first with 200 μL pipette set to 180 μL, then with 20 μL pipette set to 20 μL to remove any excess ethanol. The plate was then removed from magnet and 40 μL of RNase/DNase free molecular biology grade water (Promega, cat# P1195) from was added and mixed by pipetting. Bead were incubated with the water for one minute, the place on magnet and allowed to sit for one minute. Careful not to disturb the beads, water was carefully removed and placed in a new 0.2 mL PCR plate.

Cleaned 16S amplicons were then barcoded using dual indexes from Illumina (Nextera XT Index kit V2, Illumina cat# 15052163) PCR reactions were set up similarly to the 16S reaction described above with the following exceptions, 2.5 μL of cleaned 16S amplicons were added as the DNA template and 5 μL of each indexing primer was used. The same thermocycler conditions were used as for the 16S PCR with the following modification: only 8 cycles were run. Indexed, 16S amplicons were then cleaned following the same SPRIselect protocol detailed above, with the following modifications: beads are added at a 1:1

ratio (25 µL of beads for 25 µL reaction. Following clean-up, samples were quantified by fluorescence (DeNovix dsDNA high sensitivity assay cat# Kit-DSDNA-HIGH-2).

Sequencing

10 ng of each sample were pooled for the sequencing. The samples were first pooled by operator. Pools were quantified by Fluorescence (DeNovix dsDNA high sensitivity assay cat# Kit-DSDNA-HIGH-2) and nM of each pool was calculated. DNA pools were stored at 4 °C until sequencing. On the day of sequencing, each operator diluted the pools to 4 nM. Pools were combined in equal volume and prepared for sequencing following the MiSeq System Denature and Dilute Libraries Guide (Document # 15039740 v10, Protocol A). Denatured libraries were diluted to a final concentration of 12 pM and combined with 5 % PhiX control (V3 cat# 15017666 from Illumina). Paired-end sequencing was performed on an Illumina MiSeq with 2×300 bp reads (MiSeq Reagent Kit v3 600-cycle, cat #: MS-102-3003).

Data analysis

Adapter trimming was done as part of the Illumina MiSeq Generate FASTQ workflow. Fastq files were imported to RStudio (R version 4.1.0) for processing. Cutadapt (2.8)(25) was used for primer trimming followed by DADA2 (1.20.0)(26) with taxonomic assignment. Briefly, Cutadapt identified and trimmed primers allowing for no more than 2 mismatches with the primer sequencing. Primer trimmed sequences were then fed into the DADA2 pipeline. Reads were filtered and trimmed using the following parameters: all reads (forwards and reverse) were trimmed to 250 bp, reads could contain no Ns, maxEE (expected error) of five for both forward and reverse reads, and the default trunQ (2). Default settings for error learning, dereplicating, merging, and chimera identification were used. The R code for the initial sequence processing and the statistical analyses shown herein are publicly available at https://data.nist.gov/od/id/mds2-3092.

NIST Disclaimer

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.

Ethics approval and Consent to Participate

All work was reviewed and approved by the U. S. National Institute of Standards and Technology (NIST) Research Protections Office. This study (protocol #: MML-2019-0135) was determined to be "not human subjects research" as defined in the Common Rule (45 CFR 46, Subpart A).

Consent for Publication

Not applicable.

Availability of Data and Materials

All metagenomic sequencing results and the code used for analyses in this manuscript are available online (https://data.nist.gov/od/id/mds2-3092). Remaining units of the fecal materials used for this project are available for purchase from The BioCollective.

Competing Interests

The authors declare no competing interests.

Authors Contributions

S.P.F., S.L.S., J.G.K., J.J.F., and S.A.J. conceptualized the project and designed experiments. J.N.D. and M.E.H. analyzed samples. S.P.F. and S.L.S. analyzed data. S.P.F., S.L.S., J.G.K., and S.A.J. wrote the

564 manuscript and prepared figures. All authors approved the final manuscript except for J.J.F. who passed
565 away before the completion of the project.

566 **Acknowledgements**

567 The authors wish to acknowledge Sheng Lin-Gibson, Kirsten H. Parratt, Alshae R. Logan, and Lisa M.
568 Stabryla for critical feedback during manuscript review.

569 **References**

570 1. Simner PJ, Miller S, Carroll KC. 2018. Understanding the Promises and Hurdles of Metagenomic
571 Next-Generation Sequencing as a Diagnostic Tool for Infectious Diseases. Clinical Infectious
572 Diseases 66:778-788.
573 2. Goldberg B, Sichtig H, Geyer C, Ledeboer N, Weinstock GM. 2015. Making the Leap from
574 Research Laboratory to Clinic: Challenges and Opportunities for Next-Generation Sequencing in
575 Infectious Disease Diagnostics. Mbio 6.
576 3. Chiu CY, Miller SA. 2019. Clinical metagenomics. Nature Reviews Genetics 20:341-355.
577 4. Sczyrba A, Hofmann P, Belmann P, Koslicki D, Janssen S, Droge J, Gregor I, Majda S, Fiedler J,
578 Dahms E, Bremges A, Fritz A, Garrido-Oter R, Jorgensen TS, Shapiro N, Blood PD, Gurevich A, Bai
579 Y, Turaev D, DeMaere MZ, Chikhi R, Nagarajan N, Quince C, Meyer F, Balvociute M, Hansen LH,
580 Sorensen SJ, Chia BKH, Denis B, Froula JL, Wang Z, Egan R, Don Kang D, Cook JJ, Deltel C,
581 Beckstette M, Lemaitre C, Peterlongo P, Rizk G, Lavenier D, Wu YW, Singer SW, Jain C, Strous M,
582 Klingenberg H, Meinicke P, Barton MD, Lingner T, Lin HH, Liao YC, et al. 2017. Critical
583 Assessment of Metagenome Interpretation-a benchmark of metagenomics software. Nat
584 Methods 14:1063-1071.
585 5. Lim MY, Song EJ, Kim SH, Lee J, Nam YD. 2018. Comparison of DNA extraction methods for
586 human gut microbial community profiling. Syst Appl Microbiol 41:151-157.

587 6. Sinha R, Abu-Ali G, Vogtmann E, Fodor AA, Ren B, Amir A, Schwager E, Crabtree J, Ma S, Abnet
588 CC, Knight R, White O, Huttenhower C. 2017. Assessment of variation in microbial community
589 amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. Nature
590 Biotechnology 35:1077-1086.
591 7. Zaiko A, Greenfield P, Abbott C, von Ammon U, Bilewitch J, Bunce M, Cristescu ME, Chariton A,
592 Dowle E, Geller J, Gutierrez AA, Hajibabaei M, Haggard E, Inglis GJ, Lavery SD, Samuiloviene A,
593 Simpson T, Stat M, Stephenson S, Sutherland J, Thakur V, Westfall K, Wood SA, Wright M, Zhang
594 G, Pochon X. 2022. Towards reproducible metabarcoding data: Lessons from an international
595 cross-laboratory experiment. Molecular Ecology Resources 22:519-538.
596 8. McLaren M, Willis A, Callahan B. 2019. Consistent and correctable bias in metagenomic
597 sequencing experiments. Elife 8.
598 9. Shkoporov AN, Ryan FJ, Draper LA, Forde A, Stockdale SR, Daly KM, McDonnell SA, Nolan JA,
599 Sutton TDS, Dalmasso M, McCann A, Ross RP, Hill C. 2018. Reproducible protocols for
600 metagenomic analysis of human faecal phageomes. Microbiome 6:68.
601 10. Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P, Balamurugan R. 2020. The
602 Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients
603 12:1474.
604 11. Trutna LS, P; del Castillo, E; Moore, T; Hartley, S; Hurwitz, A. 2012. *In* Tobias PT, L (ed),
605 NIST/SEMATECH e-Handbook of Statistical Methods.
606 https://www.itl.nist.gov/div898/handbook/. doi:doi.org/10.18434/M32189.
607 12. Galyean AA, Filliben JJ, Holbrook RD, Vreeland WN, Weinberg HS. 2016. Asymmetric flow field
608 flow fractionation with light scattering detection - an orthogonal sensitivity analysis. J
609 Chromatogr A 1473:122-132.

610 13. Scott JHJ. 2007. Accuracy issues in chemical and dimensional metrology in the SEM and TEM.
611 Measurement Science and Technology 18:2755-2761.
612 14. Lee Y, Filliben JJ, Micheals RJ, Jonathon Phillips P. 2013. Sensitivity analysis for biometric
613 systems: A methodology based on orthogonal experiment designs. Computer Vision and Image
614 Understanding 117:532-550.
615 15. Forry SP, Servetas SL, Kralj JG, Soh K, Hadjithomas M, Cano R, Carlin M, de Amorim MG, Auch B,
616 Bakker MG, Bartelli TF, Bustamante JP, Cassol I, Chalita M, Dias-Neto E, Duca AD, Gohl DM,
617 Kazantseva J, Haruna MT, Menzel P, Moda BS, Neuberger-Castillo L, Nunes DN, Patel IR, Peralta
618 RD, Saliou A, Schwarzer R, Sevilla S, Takenaka IKTM, Wang JR, Knight R, Gevers D, Jackson SA.
619 2023. doi:10.1101/2023.04.28.538741.
620 16. Morton JT, Marotz C, Washburne A, Silverman J, Zaramela LS, Edlund A, Zengler K, Knight R.
621 2019. Establishing microbial composition measurement standards with reference frames. Nat
622 Commun 10:2719.
623 17. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. 2014. Unifying the
624 analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene
625 sequencing and selective growth experiments by compositional data analysis. Microbiome 2:15.
626 18. Gloor GB, Wu JR, Pawlowsky-Glahn V, Egozcue JJ. 2016. It's all relative: analyzing microbiome
627 data as compositions. Ann Epidemiol 26:322-9.
628 19. Houtman TA, Eckermann HA, Smidt H, de Weerth C. 2022. Gut microbiota and BMI throughout
629 childhood: the role of firmicutes, bacteroidetes, and short-chain fatty acid producers. Scientific
630 Reports 12.
631 20. Mariat D, Firmesse O, Levenez F, Guimaraes VD, Sokol H, Dore J, Corthier G, Furet JP. 2009. The
632 Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. Bmc Microbiology 9.

633 21. Takezawa K, Fujita K, Matsushita M, Motooka D, Hatano K, Banno E, Shimizu N, Takao T, Takada
634 S, Okada K, Fukuhara S, Kiuchi H, Uemura H, Nakamura S, Kojima Y, Nonomura N. 2021. The
635 Firmicutes/Bacteroidetes ratio of the human gut microbiota is associated with prostate
636 enlargement. Prostate 81:1287-1293.
637 22. Janssens Y, Nielandt J, Bronselaer A, Debunne N, Verbeke F, Wynendaele E, Van Immerseel F,
638 Vandewynckel YP, De Tre G, De Spiegeleer B. 2018. Disbiome database: linking the microbiome
639 to disease. BMC Microbiol 18:50.
640 23. McLaren MR, Nearing JT, Willis AD, Lloyd KG, Callahan BJ. 2022.
641 doi:10.1101/2022.08.19.504330.
642 24. Sergaki C, Anwar S, Fritzsche M, Mate R, Francis RJ, MacLellan-Gibson K, Logan A, Amos GCA.
643 2022. Developing whole cell standards for the microbiome field. Microbiome 10:123.
644 25. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.
645 EMBnetjournal 17.
646 26. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-
647 resolution sample inference from Illumina amplicon data. Nat Methods 13:581-3.

648